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Attorney Docket No

15060-00004

First Named Inventor or Application Identifier

Richard W Gross

Title

CALCIUM INDEPENDENT PHOSPHOLIPASE A₂
POLYNUCLEOTIDES AND POLYPEPTIDES AND METHODS
THEREFOR

Express Mail Label No

EL319731036US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

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1. ☒ Fee Transmittal Form (e.g., PTO/SB/17)
(Submit an original, and a duplicate for fee processing)6 ☐ Microfiche Computer Program (Appendix)2. ☒ Specification [Total Pages] **55**
(Preferred arrangement set forth below)7. Nucleotide and/or Amino Acid Sequence Submission
(If applicable, all necessary)

- Descriptive title of the Invention
- Cross References to Related Applications
- Statement Regarding Fed sponsored R & D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings (if filed)
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

a ☐ Computer Readable Copyb ☐ Paper Copy (identical to computer copy)c ☐ Statement verifying identity of above copies3. ☒ Drawing(s) (35 USC 113) [Total Sheets] **32**4. Oath or Declaration [Total Pages] ☐a. ☐ Newly executed (original or copy)b. ☐ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
[Note Box 5 below]i ☐ **DELETION OF INVENTOR(S)**
Signed statement attached deleting inventor(s) named
in the prior application, see 37 CFR 1.63(d)(2) and
1.33(b).5. ☐ Incorporation by Reference (useable if Box 4b is checked)
The entire disclosure of the prior application, from which a copy of the
oath or declaration is supplied under Box 4b, is considered as being part
of the disclosure of the accompanying application and is hereby
incorporated by reference therein**ACCOMPANYING APPLICATION PARTS**8 ☐ Assignment Papers (cover sheet & document(s))9 ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(when there is an assignee)10 ☐ English Translation Document (if applicable)11 ☐ Information Disclosure ☐ Copies of IDS
Statement (IDS)/PTO-1449 Citations12 ☐ Preliminary Amendment13 ☒ Return Receipt Postcard (MPEP 503)
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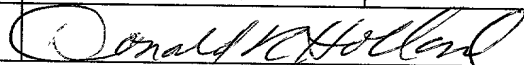
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15060-00004

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I certify that the attached utility patent application of **RICHARD W. GROSS and DAVID J. MANCUSO, for CALCIUM INDEPENDENT PHOSPHOLIPASE A₂ POLYNUCLEOTIDES AND POLYPEPTIDES AND METHODS THEREFOR** including:

- Fifty-one (51) pages of specification; Three (3) pages of claims; one (1) page of abstract
- Declaration and Power of Attorney, submitted for identification of inventors only (2 pgs)
- Thirty-two (32) sheets of drawings
- Patent Application Transmittal (1 page)
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CALCIUM INDEPENDENT PHOSPHOLIPASE A₂ γ
POLYNUCLEOTIDES AND POLYPEPTIDES AND
METHODS THEREFOR

REFERENCE TO GOVERNMENT GRANT

This invention was made with U.S. government support under National Institute of Health Grant Numbers 1 PO1 HL 57278-02, 2 RO2 HL 41250-08A1, and 5RO1 AA11094. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

(1) Field of the Invention

This invention relates to phospholipases and, more particularly, to novel calcium-independent phospholipase A₂ γ polypeptides and to nucleic acids encoding the polypeptides, as well as to methods of making and using the polypeptides and nucleic acids.

(2) Description of the Related Art

The function of complex biological organisms relies on the meticulous control of cellular activity, including the close regulation of cell growth, proliferation, and function. The family of enzymes known as the phospholipases A₂ has been implicated in the control of cellular activity by catalyzing the esterolytic cleavage of fatty acids from phospholipids, thereby regulating the release of lipid second messengers, cellular growth factors, and the properties of the cellular membrane. (Samuelsson et al., *Annu. Rev. Biochem.* 47:997-1029, 1978; Moolenaar, W.H., *Curr. Opin. Cell. Biol.* 7:203-10, 1995). In particular, by controlling the production of second messengers such as arachidonic acid and its biologically active eicosanoid metabolites, the phospholipases A₂ are involved in modulating such processes as cellular growth programs, inflammation, vascular tone and ion channel function. (Needleman et al., *Annu. Rev. Biochem.* 55:69-102, 1986).

However, the phospholipases A₂ are a broad family of enzymes with varying kinetic and physical properties, and distinct functions. Early research focused on distinguishing broad classes of the enzymes within the larger family. Several classes

were distinguished using *in vitro* activity assays, and are based on the dependence of enzyme activity on the presence calcium ion. (See e.g., Demel et al., *Biochim. Biophys. Acta* 406:97-107, 1975). Thus, one class, the secretory phospholipases A₂ are distinguished by an obligatory dependence on high (millimolar) concentrations of calcium ion, as well as low molecular weights (14-18 kDa) and relative heat stability. (Demel et al., *supra*; Tischfield, J.A., *J. Biol. Chem.* 272:17247-50, 1997). The activity of a second class, the calcium-facilitated phospholipases A₂, is facilitated by the presence of nanomolar concentrations of calcium ion, but the presence of the calcium ion is not obligatory. (Loeb et al., *J. Biol. Chem.* 261:10467-70, 1986; Kramer et al., *Biochim. Biophys. Acta* 878:394-403; Glover et al., *J. Biol. Chem.* 270:15359-67, 1986). A third class of enzymes is entirely calcium-independent in *in vitro* studies, and is also distinguished by a finely tuned inhibition by (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (BEL). (Wolf et al., *J. Biol. Chem.* 260:7295-303; Hirashima et al., *J. Neurochem.* 59:708-14; Lehman et al., *J. Biol. Chem.* 268:20713-16).

Application of molecular biological techniques has provided some insights into the structure and function of founding members in each class of phospholipases A₂, and has provided a further basis for distinguishing among the classes. (See, e.g. Demel et al, *supra*; Evenberg et al., *J. Biol. Chem.* 252: 1189-96, 1977; Tischfeld, J.A., *J. Biol. Chem.* 272: 17247-50, 1997). For example, members of the secretory phospholipases A₂ use a calcium ion to polarize the *sn*-2-carbonyl for attack by a histidine-activated H₂O molecule, while the intracellular phospholipases use a nucleophilic serine. The calcium-facilitated phospholipases A₂ have a GXSGS consensus lipase motif, in contrast to the iPLA₂ group which has a GXSTG consensus motif. The calcium-independent phospholipases A₂ are also distinguished by a consensus sequence for nucleotide binding. (Andrews et al., *Biochem. J.* 252:199-206, 1988; Tang et al., *J. Biol. Chem.* 272:8567-75, 1998). These findings have clearly boosted progress toward identifying the polypeptides responsible for catalyzing the synthesis of the eicosanoid metabolites and toward understanding the regulatory mechanisms of phospholipases A₂ that are involved in normal and disease states.

The more recent development of intense genome sequencing efforts have produced partial sequence data on the phospholipases, and related structural insights. For

example, two new calcium-facilitated phospholipases have recently been described based on data from protein and nucleotide data bases. (Underwood et al., *J Biol. Chem.* 273: 21926-32, 1998; Pickard et al., *J. Biol. Chem.* 274: 8823-31, 1999).

Further, during sequencing of the long arm of chromosome 7 in the Human Genome Sequencing Project, a predicted protein product of 40kDa was identified. The polypeptide contained two ~10 amino acid segments homologous to the lipase and nucleotide-binding consensus sequences described for the founding members of the iPLA₂ family. (Tang et al., *supra*).

Some earlier work has suggested a connection between some types of phospholipases and certain disease conditions. For example, intensive study of reperfusion injury in myocardial tissue has led to the hypothesis that pathology is ultimately generated because of membrane phospholipid breakdown attributable to activation of myocardial phospholipase A₂ activity. (See e.g. Van der Vusse et al., Hydrolysis of phospholipids and cellular integrity, In: H.M. Piper (ed.) *Pathophysiology of Severe Ischemic Myocardial injury*, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1990, 167-93). Furthermore, calcium-dependent and calcium-independent phospholipase A₂ activities have also been found to be present in human cerebral cortex. Some reports have suggested a possible link between the activity of phospholipases A₂, both calcium-dependent calcium independent, and cortical degenerative diseases such as Alzheimer's disease. (For reviews, see e.g. Farooqui et al., *Neurochem. Int.* 30: 517-22, 1997; Farooqui et al., *Brain Res. Bull.* 49: 139-53, 1999).

Certain inhibitors of phospholipases A₂ have been identified as possible therapeutic candidates for treating PLA₂-mediated diseases. For example, fatty acid trifluormethyl ketones, bromoenol lactone, methyl arachidonyl fluorophosphonate, bezenesulfonamides and other specific inhibitors of phospholipases A₂ have been shown to decrease PLA₂ activity and all have been considered for treating inflammatory diseases thought to be mediated by PLA₂. (See e.g. Farooqui et al, 1999, *supra*). Nevertheless, as noted above, the phospholipases A₂, as well as the iPLA₂ subfamily itself, are a heterogeneous group of enzymes, with differing molecular weights, substrates, and responses to inhibitors. Because of this, the development of

agents for treating diseases mediated by these compounds is ideally based upon the structure and functional characteristics of the particular iPLA₂ involved in the disease process. Thus, it is crucial to identify and characterize the phospholipases A₂ in order to develop new drugs specific for those phospholipases A₂.

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BRIEF SUMMARY OF THE INVENTION

Accordingly, the inventors herein have succeeded in discovering a novel phospholipase A₂, referenced herein as calcium-independent phospholipase A₂ (iPLA₂), and nucleic acids encoding iPLA₂. The new calcium-independent phospholipase A₂ is involved with fatty acid β -oxidation in the peroxisomal compartment and is believed to be involved in certain disease processes. More specifically, the present invention is based on the collective work reported herein which identifies the complete genomic organization, nucleic acid sequence, and *sn*-2-lipase activity of a novel calcium-independent membrane-associated phospholipase A₂.

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Thus, in one embodiment, the invention is directed to an isolated nucleic acid molecule comprising a iPLA₂ polynucleotides. In one aspect of this embodiment, the iPLA₂ polynucleotides encode an iPLA₂ polypeptide. The iPLA₂ polypeptide catalyzes cleavage of fatty acids from the *sn*-2-position of phospholipids. Preferred sequences are as set forth in SEQ ID NO: 1 and SEQ ID NO: 2 which represent the amino acid sequences corresponding to alternative splice variants of iPLA₂. Splice variants, identified herein as gamma 1 and gamma 2, involve splice sites 5' to the start codon so that both splice variants encode the same iPLA₂ polypeptide. Splice variant gamma 3, however, utilizes a start codon 5' to that of gamma 1 and gamma 2 to encode a longer polypeptide. SEQ ID NO:3 constitutes the cDNA of the coding region and SEQ ID NO:1 constitutes the encoded polypeptide of alternative splice variants gamma 1 and gamma 2. SEQ ID NO:4 constitutes the cDNA of the coding region of splice variant gamma 2 and SEQ ID NO:2 constitutes the encoded polypeptide.

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The present invention is also directed to iPLA₂ polynucleotides and fragments thereof which specifically hybridize under stringent conditions to the complements of

the coding regions of iPLA₂ γ polynucleotides, the coding regions being set forth in SEQ ID NO:3 and SEQ ID NO:4 and the corresponding complements, SEQ ID NO:4 and SEQ ID NO:6, respectively. In another aspect of this embodiment, the present invention is directed to iPLA₂ γ polynucleotides comprising polynucleotides having at least about 90% identity with SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO: 6 and, preferably, such polynucleotides comprise SEQ ID NO: 3, SEQ ID NO:4, SEQ ID NO: 5, or SEQ ID NO: 6.

In another aspect of this embodiment, the present invention is also directed to vectors and cells comprising an a polynucleotide encoding an iPLA₂ γ polynucleotide. In addition, the present invention encompasses antisense compounds which specifically hybridizes to SEQ ID NO: 3 or SEQ ID NO:4.

In one particular aspect of this embodiment, the present invention is directed to a fragment of an iPLA₂ γ polynucleotide comprising an iPLA₂ γ repressor binding site. Preferably, the polynucleotide comprises SEQ ID NO:7, 5'-TGATTTCACGTTTAGCTCAATT-3'.

In another embodiment, the present invention is directed to an isolated polypeptide comprising a phospholipase A₂ γ . The isolated polypeptide catalyzes cleavage of fatty acids from the *sn*-2-position of phospholipids. In one aspect of this embodiment, the polypeptide has at least about 90% identity with SEQ ID NO:1 or SEQ ID NO: 2 and, more preferably, the polypeptide comprises SEQ ID NO:1 or SEQ ID NO: 2. In another aspect of this embodiment, the present invention comprises a conservatively substituted variant of SEQ ID NO:1 or SEQ ID NO:2. The present invention also encompasses antibodies capable of binding to a phospholipase A₂ γ polypeptide.

Another embodiment of the present invention constitutes a method of treating inflammation in a patient. The method comprises treating the patient in to decrease calcium-independent phospholipase A₂ γ activity in the patient. It is contemplated that patients in need of such treatment include those patients suffering from Alzheimer's disease, myocardial ischemia, or myocardial infarction. Preferably, the method comprises administering to the patient a phospholipase A₂ γ translational repressor molecule. In another aspect of this invention, the method can also comprise

administering to the patient an antisense sequence which specifically hybridizes to SEQ ID NO: 3 or SEQ ID NO: 4.

In another embodiment, the present invention is directed to a method of increasing fatty acid utilization in a patient in need thereof. The method comprises increasing iPLA₂γ activity in the patient. Patients in need of such treatment include those patients suffering from diabetes as well as those patients suffering from obesity. Preferably, the method comprises administering to the patient a substance which blocks translational repression of iPLA₂γ. In another aspect of this invention, the method can also comprise administering to the patient an iPLA₂γ polypeptide or a polynucleotide encoding said iPLA₂γ polypeptide.

Another embodiment of the present invention is directed to a method for measuring activity of a phospholipase A₂γ polypeptide of cells in a biological sample. The method comprises introducing into the sample a phospholipid substrate for cleavage of fatty acids by the phospholipase A₂γ, wherein the phospholipase A₂γ cleaves fatty acid from the *sn*-2-position of the phospholipid substrate. The method further comprises measuring cleavage of the phospholipid substrate and/or quantifying the release of fatty acid from the substrate.

The present invention is also directed to an assay method for identifying substances which modulate iPLA₂γ expression in a cell. The method comprises contacting a candidate substance with cells comprising a promoter sequence operably linked to an iPLA₂γ repressor binding site and a reporter gene and measuring expression of the reporter gene. By iPLA₂γ repressor binding site it is meant that portion of nucleotide sequence of an iPLA₂γ polynucleotide to which one or more endogenous substances within a cell bind to regulate translation of that iPLA₂γ polynucleotide. Preferably, the iPLA₂γ repressor binding site comprises SEQ ID NO:7. The reporter gene, preferably, encodes an enzyme capable of being detected by a colorimetric, fluorimetric or luminometric assay such as, for example, a reporter sequence encoding a luciferase. In a particularly preferred embodiment, the promoter sequence is a baculovirus promoter sequence and the cells are Sf9 cells.

The present invention also encompasses genetically engineered cells capable of identifying substances which modulate iPLA₂γ expression in a cell. The cells comprising a promoter operably linked to an iPLA₂γ repressor binding site and a

reporter gene. Preferably, the iPLA₂γ repressor binding site comprises SEQ ID NO:7. The reporter gene, preferably, encodes an enzyme capable of being detected by a colorimetric, fluorimetric or luminometric assay such as, for example, a reporter sequence encoding a luciferase. In a particularly preferred embodiment, the promoter sequence is a baculovirus promoter sequence and the cells are Sf9 cells.

The present invention also includes a method for identifying substances which modulate iPLA₂γ expression. The method comprising contacting a candidate substance with cells capable of expressing iPLA₂γ or a fragment thereof and measuring the expression of iPLA₂γ or fragment thereof by the cells, wherein a level of expression greater or less than that in absence of the substance indicates activity in modulating iPLA₂γ expression.

Among the several advantages found to be achieved by the present invention, therefore, may be noted the provision of a novel phospholipase A₂γ, iPLA₂γ, which catalyzes the release of fatty acids from the *sn*-2-position of phospholipid substrate; the provision of methods for identifying a substance which represses and thus controls iPLA₂γ expression in a sample; the provision of methods for treating diseases involving inflammatory processes, inflammation or fatty acid utilization; and the provision of methods for determining the presence and level of activity or expression of iPLA₂γ in a sample.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the iPLA₂γ cDNA sequence (SEQ ID NO:8) which contains the coding region for the 88kDa iPLA₂γ polypeptide along with the corresponding amino acid sequence (SEQ ID NO:9).

Figure 2 illustrates three alternative splice variants of the iPLA₂γ gene.

Figure 3 illustrates the 5' portion of three alternative splice variants, identified as 1 (SEQ ID NO:10), 2 (SEQ ID NO:11) and 3 (SEQ ID NO:12) along with amino terminal portion of the encoded polypeptides corresponding to splice variants 1 and 2 (SEQ ID NO:13) and splice variant 3 (SEQ ID NO:14).

Figure 4 illustrates the full-length 88kDa polypeptide (SEQ ID NO:1) and the cDNA encoding the polypeptide (SEQ ID NO:3) along with sense primer, M444 (SEQ ID NO:15) and reverse primer, M458 (SEQ ID NO:16) used in the PCR amplification of the 88kDa polypeptide.

5 Figure 5 illustrates the 77kDa truncated polypeptide starting at amino acid 101 and nucleotide 301 (SEQ ID NO:17) and the cDNA encoding the polypeptide (SEQ ID NO:18) along with sense primer, m533 (SEQ ID NO:19) and reverse primer, M458 (SEQ ID NO:20) used in the PCR amplification of the 74kDa polypeptide.

10 Figure 6 illustrates the 74kDa truncated polypeptide starting at amino acid 122 and nucleotide 364 (SEQ ID NO:21) and the cDNA encoding the polypeptide (SEQ ID NO:22) along with sense primer, m534 (SEQ ID NO:23) and reverse primer, M458 (SEQ ID NO:24) used in the PCR amplification of the 74kDa polypeptide.

15 Figure 7 illustrates the 63kDa truncated polypeptide starting at amino acid 221 and nucleotide 661 (SEQ ID NO:25) and the cDNA encoding the polypeptide (SEQ ID NO:26) along with sense primer, m530 (SEQ ID NO:27) and reverse primer, M458 (SEQ ID NO:28) used in the PCR amplification of the 74kDa polypeptide.

20 Figure 8 is a schematic illustration mapping expressed sequence tags overlapping the full-length sequence of iPLA₂γ.

Figure 9 illustrates the analysis of PCR amplified human iPLA₂γ on agarose gel.

25 Figure 10 is a schematic representation of the strategy used for sequencing a PCR product encoding iPLA₂γ.

Figure 11 is a hydropathy plot of the deduced amino acid sequence of human iPLA₂γ.

Figure 12 is a Northern blot of human iPLA₂γ mRNA obtained from multiple tissue samples.

Figure 13 is a schematic illustration of the genomic organization of the human iPLA₂γ gene.

5 Figure 14 shows the results of gel electrophoresis and autoradiographic visualization of the *in vitro* translation products of iPLA₂γ.

Figure 15 shows the results of Western analysis of iPLA₂γ expression in Sf9 cells.

10 Figure 16 is a graph of an initial rate analysis of the iPLA₂ activity of iPLA₂γ.

Figure 17 is a substrate selectivity profile of the iPLA₂ activity of iPLA₂γ.

Figure 18 is a graph of inhibition of iPLA₂ activity of iPLA₂γ by BEL.

15 Figure 19 shows the results of Western analysis of expression of truncated iPLA₂γ constructs in Sf9 cells.

Figure 20 is a schematic representation of iPLA₂γ translational repression constructs.

Figure 21 is a schematic representation of the pFASTBAC vector containing gamma 23mer sequences and luciferase coding sequence.

20 Figure 22 is a schematic representation of baculovirus promoter constructs containing gamma 23mer sequences and luciferase coding sequence.

Figure 23 shows phosphorylated oligo pairs for sequences between nucleotides 364 and 455 for use in translational repression of iPLA₂γ in the luciferase expression system.

Figure 24 is a bar graph showing luciferase assay results showing the relative levels of expression of three different iPLA₂γ constructs in Sf9 cells.

DETAILED DESCRIPTION OF THE INVENTION

Thus, the present invention is based upon the identification, isolation and sequencing of the novel calcium-independent phospholipase A₂, iPLA₂γ. The new iPLA₂γ is both membrane-associated and highly sensitive to inhibition by (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (BEL), which distinguishes the new iPLA₂γ from previously reported membrane-associated phospholipases A₂. Prior to this invention, iPLA₂γ was unknown and had not been distinguished as a biologically active substance discrete from known calcium-independent phospholipases A₂, nor had it been isolated in pure form and its putative sequenced determined.

Reference to iPLA₂γ or iPLA₂γ polypeptides herein is intended to be construed to include the polypeptides corresponding to coding regions of three spliced variants identified herein. The amino acid sequences of the polypeptides are as set forth in SEQ ID NO:1 and SEQ ID NO:2. The coding portion of the cDNA of the splice variants are as set forth in SEQ ID NO:3 (splice variants gamma 1 and gamma 2) and SEQ ID NO:4 (splice variant gamma 3) (see Figures 2-4). The iPLA₂γ polypeptides within the present invention are also intended to include iPLA₂γ's of any origin which are substantially identical to and which are biologically equivalent to the iPLA₂γ polypeptides characterized and described herein. Such substantially identical iPLA₂γ's may be native to any tissue or species and, similarly, biological activity can be characterized in any of a number of biological assay systems. The term "biologically equivalent" is intended to mean that the compositions of the present invention are capable of demonstrating some or all of the same *sn*-2-lipase activity, membrane localization and sensitivity to BEL inhibition, not necessarily to the same degree as the iPLA₂γ isolated herein as described in the examples below.

By "substantially identical" it is meant that the degree of identity of human iPLA₂ γ to the iPLA₂ γ from any species is greater than that between the iPLA₂ γ and any previously reported member of the iPLA₂ family. Sequence identity or percent identity is intended to mean the percentage of same residues between two sequences when the two sequences are aligned by methods well known to those skilled in the art (for example, see Higgins et al, *Cabios* 8:189-191, 1992; Dayhoff et al., in *ATLAS OF PROTEIN SEQUENCE AND STRUCTURE*, Dayhoff, Ed., NDRF, Washington, Vol. 5, suppl. 3, p. 345, 1978).

Polypeptides considered to be included within the term iPLA₂ polypeptides also includes conservatively substituted variants of SEQ ID NO:1 or SEQ ID NO:2. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups include: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine, glutamic acid-aspartic acid, leucine-methionine; glutamine-histidine.

The iPLA₂ γ polynucleotides of the present invention include those nucleic acid molecules, both DNA and RNA, which encode the iPLA₂ γ polypeptides. Such iPLA₂ γ polypeptides include, in particular, the coding regions of splice variants as set forth in SEQ ID NO:3 (splice variants gamma 1 and gamma 2) and SEQ ID NO:4 (splice variant gamma 3) (see Figures 2-4) as well as their complements, SEQ ID NO:4 and SEQ ID NO:6, respectively. Also included are cDNA sequences which show at least 20% identity with the cDNA sequences of SEQ ID NOS:3, 4, 5 or 6 as defined above and sequences which specifically hybridize to SEQ ID NOS:3, 4, 5 or

6. By specifically hybridizing to said sequences, it is meant that a given sequence hybridizes under high stringency conditions. The stringency of hybridization is determined by a number of factors during hybridization and during the washing procedure, including temperature; ionic strength, length of time and concentration of formamide. These factors are outlined in, for example, Sambrook et al. (Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). High stringency conditions serve to prevent false positives, that is the hybridization and apparent detection of iPLA₂γ polynucleotide sequences when in fact an intact and functioning iPLA₂γ polynucleotide is not present.

Probes and primers which are capable of hybridizing to an iPLA₂γ sequence are also included within the present invention. Probes can be oligonucleotides of from about 25 to about 100 nucleotides in length which specifically hybridize to a target sequence. Primers within the scope of the present invention are typically short strands of DNA or RNA having a length of at least from about 6 to about 8 nucleotides, more preferably about 12 nucleotides, still more preferably about 15 nucleotides, and most preferably about 20 to about 30 nucleotides or greater in length. Such nucleic acid sequences hybridize to the target region. The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand.

Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

The present invention also thus includes fragments and truncated polynucleotides which can be used in modulating the translational regulation of

iPLA₂γ, and in the identification of naturally occurring repressor substances within the cell. Thus, by cloning of portions of the wild type iPLA₂γ polynucleotide into a vector one can identify those portions of the sequence involved in translational regulation within the cell. These polynucleotide portions of the iPLA₂γ sequence can bind to

5 naturally occurring repressor substances in a cell and serve as binding ligands to the repressor substance. Such binding can block the decrease in iPLA₂γ polynucleotide expression action that would otherwise occur if the repressor substance were free to bind to the iPLA₂γ polynucleotide. In addition, the binding to a repressor substance by the polynucleotides can serve as a basis for identifying and isolating new compounds

10 which prevent the binding of the repressor substance to iPLA₂γ polynucleotides. Such binding to the repressor substance produces a de-repressor action to allow an increased expression of iPLA₂γ polynucleotides.

The present invention also includes treatment of certain diseases using iPLA₂γ, protein products thereof, or a translational repressor molecule thereof. Based

15 on its genomic characterization and biological activity, iPLA₂γ is a likely candidate for involvement in a number of disease processes. Without being bound to a particular theory, the inventors herein have discovered that iPLA₂γ is a likely candidate for involvement in Alzheimer's disease because its *sn*-2-lipase activity regulates the release of lipid second messengers. Such second messengers include

20 arachidonic acid and its eicosanoid metabolites which are prominent lipid second messengers for inflammatory processes. (See e.g. Needleman et al., *supra*). It has been previously established that the neural damage underlying Alzheimer's disease ultimately results from a final common pathway involving precipitation of amyloid protein in a plaque formation that initiates lipid inflammation and produces dystrophic

25 changes in the affected neurons. The amyloid plaques of Alzheimer's disease are a nidus for localized inflammation, and the neural dystrophic process in Alzheimer's disease is characterized by the presence of several biochemical markers of inflammation, and inflammation may be the proximal cause of synaptic loss during neural dystrophy. (See e.g. Farooqui et al., *supra*). Thus, the inventors herein have, in

30 identifying and characterizing iPLA₂γ, also succeeded in identifying iPLA₂γ as a new

candidate for mediating inflammatory processes involved in Alzheimer's disease. Treatment for Alzheimer's disease would thus involve any treatment that decreases the activity of iPLA₂γ, especially in brain regions affected by Alzheimer's disease.

5 The iPLA₂γ polypeptides of the present invention can also be used in
the treatment of atherosclerotic heart disease, which represents a major cause of
morbidity and mortality in the United States and other industrialized nations. In
patients with myocardial infarction, the infarct size and type (i.e. transmural or
subendocardial) profoundly affect realignment and remodeling of affected myocardial
zones, and hence affect whether the infarct results in heart failure, or merely
10 electrophysiologic dysfunction. Recent experimental results have shown that agents
that enhance the efficiency of ATP production from the available O₂ supply have a
salutary effect on the size and type of myocardial infarction. These findings implicate
the phenomenon known as oxygen wasting, and pose as therapeutic candidates any
agents that may have a beneficial effect on the efficiency of O₂ utilization by reducing
15 oxygen wasting. In normal myocardium, most fatty acid oxidation occurs in the
mitochondrial compartment, while a smaller but not insignificant portion of fatty acid
oxidation, about 3-4%, occurs in peroxisomes. In the peroxisomal compartment, fatty
acid oxidation occurs without generating reducing equivalents or ATP, thus producing
no useful energy but only heat, resulting in oxygen wasting. During myocardial
20 ischemia, mitochondrial oxidation of fatty acids is impaired to about 10% of normal
levels. Thus, the oxygen wasted in the peroxisomal compartment gains greater
significance because oxygen availability becomes rate-limiting. Therefore, reduction
in oxygen wasting in the peroxisomal compartment during myocardial ischemia is
likely to profoundly benefit myocyte preservation with attendant desirable effects on
25 the clinical sequelae of ischemia or infarct. Thus, the inventors herein have, in
identifying and characterizing iPLA₂γ, have also succeeded in identifying iPLA₂γ as a
new candidate target for limiting oxygen wasting. Treatment for myocardial ischemia
or myocardial infarction would thus also involve any treatment that decreases the
activity of iPLA₂γ, especially in ischemic heart tissue.

One approach for decreasing the activity of iPLA₂γ is by the identification and administration to the cell, a translational repressor substance which decreases translation of iPLA₂γ or administration of the nucleic acid encoding the translational repressor substance. Such repressor molecules can be identified by virtue of their binding to translational repressor binding sites identified herein.

Alternatively, iPLA₂γ activity can be decreased by treatment with iPLA₂γ antisense oligonucleotides. Such antisense oligonucleotides interact through base pairing with a specific complementary nucleic acid sequence involved in the expression of iPLA₂γ such that the expression of iPLA₂ is reduced. The iPLA₂γ antisense oligonucleotides preferably comprise a sequence containing from about 8 to about 100 nucleotides and more preferably the iPLA₂γ antisense oligonucleotides comprise from about 15 to about 30 nucleotides. The iPLA₂γ antisense oligonucleotides can also contain a variety of modifications that confer resistance to nucleolytic degradation such as, for example, modified internucleoside linkages (Uhlmann and Peyman, *Chemical Reviews* 90:543-548 1990; Schneider and Banner, *Tetrahedron Lett.* 31:335, 1990), modified nucleic acid bases and/or sugars and the like.

Agents that regulate or control peroxisomal β-oxidation are also potentially useful in controlling the related diseases of obesity, hyperlipidemia and diabetes, which represent major health problems in the United States and other industrialized nations. Obesity and hyperlipidemia are primary features of Syndrome X, which identifies those at high risk for developing insulin-resistant (Type II) diabetes. Obesity and high serum lipid levels also predispose individuals to developing atherosclerosis and hypertension. Accordingly, agents that decrease body weight, serum lipid levels or the insulin-resistance characterizing Type II diabetes, are avidly sought. One type of such agents operates by increasing caloric expenditure or fatty acid β-oxidation, and significant reductions in body weight can be achieved with only small increases (about 2-3%) in caloric expenditure. Since fatty acid β-oxidation in peroxisomes wastes oxygen, producing only heat, and no energy in the form of NADH or ATP, an agent that thus increases caloric expenditure by increasing

peroxisomal fatty acid β -oxidation, directly or indirectly, has potentially major beneficial effects in the treatment of obesity, hyperlipidemia, diabetes, atherosclerosis and hypertension. Thus, the inventors herein have, in identifying and characterizing iPLA₂ γ , also succeeded in identifying iPLA₂ γ as a new candidate target for increasing caloric expenditure by increasing peroxisomal fatty acid β -oxidation. Accordingly, treatment for the related diseases of diabetes, obesity, and hyperlipidemia would thus also involve any treatment that increases the activity of iPLA₂ γ , especially in Type II diabetic or obese individuals.

The present invention, therefore, also includes therapeutic or pharmaceutical compositions comprising iPLA₂ γ polypeptides and polynucleotides as well as a blocker of iPLA₂ γ translational repressor molecule, in an effective amount for treating patients with inappropriately low levels of iPLA₂ γ . Such diseases include but are not limited to Type II diabetes, obesity and hyperlipidemia. The treatment can comprise administering a therapeutically effective amount of iPLA₂ γ polynucleotide or iPLA₂ γ protein or a blocker of iPLA₂ γ translational repression.

The therapeutic or pharmaceutical compositions of the present invention can be administered by any suitable route known in the art including for example intravenous, subcutaneous, intramuscular, transdermal, intrathecal or intracerebral. Administration can be either rapid as by injection or over a period of time as by slow infusion or administration of slow release formulation.

IPLA₂ γ can also be linked or conjugated with agents that provide desirable pharmaceutical or pharmacodynamic properties. For example, iPLA₂ γ can be coupled to any substance known in the art to promote penetration or transport across the blood-brain barrier such as an antibody to the transferrin receptor, and administered by intravenous injection (See for example, Friden et al., *Science* 259:373-377, 1993). Furthermore, iPLA₂ γ can be stably linked to a polymer such as polyethylene glycol to obtain desirable properties of solubility, stability, half-life and other pharmaceutically advantageous properties. (See for example Davis et al. *Enzyme Eng* 4:169-73, 1978; Burnham, *Am J Hosp Pharm* 51:210-218, 1994).

The compositions are usually employed in the form of pharmaceutical preparations. Such preparations are made in a manner well known in the pharmaceutical art. A preferred preparation utilizes a vehicle of physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers such as physiological concentrations of other non-toxic salts, five percent aqueous glucose solution, sterile water or the like may also be used. It may also be desirable that a suitable buffer be present in the composition. Such solutions can, if desired, be lyophilized and stored in a sterile ampoule ready for reconstitution by the addition of sterile water for ready injection. The primary solvent can be aqueous or alternatively non-aqueous. Calcium-independent phospholipase A₂γ can also be incorporated into a solid or semi-solid biologically compatible matrix that can be implanted into tissues requiring treatment.

The carrier can also contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmaceutically acceptable excipients for modifying or maintaining release or absorption or penetration across the blood-brain barrier. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dosage or multi-dose form or for direct infusion into the cerebrospinal fluid by continuous or periodic infusion. Dose administration can be repeated depending upon the pharmacokinetic parameters of the dosage formulation and the route of administration used.

It is also contemplated that certain formulations containing iPLA₂γ are to be administered orally. Such formulations are preferably encapsulated and formulated with suitable carriers in solid dosage forms. Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, gelatin, syrup, methyl cellulose, methyl- and propylhydroxybenzoates, talc, magnesium, stearate, water, mineral oil, and the like. The formulations can additionally include lubricating agents, wetting agents,

emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide rapid, sustained, or delayed release of the active ingredients after administration to the patient by employing procedures well known in the art. The formulations can also contain

5 substances that diminish proteolytic degradation and promote absorption such as, for example, surface active agents.

The specific dose is calculated according to the approximate body weight or body surface area of the patient or the volume of body space to be occupied. The dose will also be calculated dependent upon the particular route of administration

10 selected. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those of ordinary skill in the art. Such calculations can be made without undue experimentation by one skilled in the art in light of the activity disclosed herein in assay preparations of target cells. Exact dosages are determined in conjunction with standard dose-response studies. It will be

15 understood that the amount of the composition actually administered will be determined by a practitioner, in the light of the relevant circumstances including the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the chosen route of administration.

20 In a number of circumstances it would be desirable to determine the levels of containing iPLA₂γ in a patient sample. The term "detection" as used herein in the context of detecting the presence of iPLA₂γ in a patient is intended to include the determining of the amount of iPLA₂γ or the ability to express an amount of iPLA₂γ in a patient, the distinguishing of iPLA₂γ from other phospholipases A₂, the estimation

25 of prognosis in terms of probable outcome of a degenerative disease and prospect for recovery, the monitoring of iPLA₂γ levels over a period of time as a measure of status of the condition, and the monitoring of iPLA₂γ levels for determining a preferred therapeutic regimen for the patient.

To detect the presence of iPLA₂γ in a patient, a sample is obtained from the patient. The sample can be a tissue biopsy sample or a sample of blood, plasma, serum, CSF or the like. Samples for detecting iPLA₂γ can be taken from any of these tissues. To detect the presence of iPLA₂γ, an enzymatic analysis or Western blot analysis can be performed.

The term "probe" as used herein refers to a structure comprised of a polynucleotide which forms a hybrid structure with a target sequence, due to complementarity of probe sequence with a sequence in the target region. Oligomers suitable for use as probes may contain a minimum of about 8-12 contiguous nucleotides which are complementary to the targeted sequence and preferably a minimum of about 20, more preferably a minimum of about 25, still more preferably a minimum of about 30, yet still more preferably a minimum of about 40, and most preferably a minimum of about 50. Calcium-independent A₂γ gene probes of the present invention can be DNA or RNA oligonucleotides and can be made by any method known in the art such as, for example, excision, transcription or chemical synthesis. Probes may be labeled with any detectable label known in the art such as, for example, radioactive or fluorescent labels or enzymatic marker. Labeling of the probe can be accomplished by any method known in the art such as by PCR, random priming, end labeling, nick translation or the like. One skilled in the art will also recognize that other methods not employing a labeled probe can be used to determine the hybridization. Examples of methods that can be used for detecting hybridization include Southern blotting, fluorescence in situ hybridization, and single-strand conformation polymorphism with PCR amplification.

Hybridization is typically carried out at 25.- 45. °C., more preferably at 32.degree.-40.degree. C. and more preferably at 37.degree.-38.degree. C. The time required for hybridization is from about 0.25 to about 96 hours, more preferably from about one (1) to about 72 hours, and most preferably from about 4 to about 24 hours.

The PCR method for labeling probes or amplifying target sequences uses primers that flank or lie within the target, here iPLA₂γ, gene. The PCR method is

well known in the art. Briefly, this method is performed using two oligonucleotide primers which are capable of hybridizing to the nucleic acid sequences flanking a target sequence that lies within a iPLA₂ γ gene and amplifying the target sequence. The terms "oligonucleotide primer" as used herein refers to a short strand of DNA or RNA ranging in length from about 8 to about 50 bases. The upstream and downstream primers are typically from about 20 to about 30 base pairs in length and hybridize to the flanking regions for replication of the nucleotide sequence. The polymerization is catalyzed by a DNA-polymerase in the presence of deoxynucleotide triphosphates or nucleotide analogs to produce double-stranded DNA molecules. The double strands are then separated by any denaturing method including physical, chemical or enzymatic. Commonly, the method of physical denaturation is used involving heating the nucleic acid, typically to temperatures from about 80.degree. C. to 105.degree. C. for times ranging from about 1 to about 10 minutes. The process is repeated for the desired number of cycles.

The primers are selected to be substantially complementary to the strand of DNA being amplified. Therefore, the primers need not reflect the exact sequence of the template, but must be sufficiently complementary to selectively hybridize with the strand being amplified. Preferably, primers are at least 10 nucleotides long, more preferably at least 15 nucleotides long, still more preferably at least 20 nucleotides long, still more preferably at least 25 nucleotides long, still more preferably at least 30 nucleotides long, still more preferably at least 40 nucleotides long, and most preferably at least 50 nucleotides long. After PCR amplification, the DNA sequence comprising iPLA₂ or a fragment thereof is then directly sequenced and analyzed.

Exemplary embodiments of the invention are described in the following examples. Other alternative embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

The following methods were used in the examples below. Specific materials used in the work reported herein were as described herein, but those skilled in the art will recognize that many suitable alternative materials are readily available from commercial suppliers. [α - 32 P]dCTP (6000Ci/mmol) and ECL detection reagents were purchased from Amersham Pharmacia Biotech. A human heart cDNA library was purchased from Stratagene, Inc. Human heart Marathon cDNA, QuickClone human skeletal cDNA, and human MTN multiple tissue Northern blots were purchased from CLONTECH. For PCR, a Perkin-Elmer Thermocycler was used, and all PCR reagents were purchased from PE Biosystems. The pGEM-T vector and TnT Quick Coupled Transcription/Translation System were purchased from Promega. Vector pcDNA1.1 was purchased from Invitrogen. Culture media, Cellfectin and LipofectAMINE reagents for transfection of baculovirus vectors, and competent DH110Bac *Escherichia coli* cells were purchased from Life Technologies, Inc., and used according to the manufacturer's protocol. QIAfilter plasmid kits and QIAquick gel extraction kits were obtained from Qiagen, Inc. Keyhole limpet hemocyanin was obtained from Pierce. L- α -1-dipalmitoyl-2-[1- 14 C]phosphatidylcholine, L- α -1-palmitoyl-2-[1- 14 C]oleoyl phosphatidylcholine, L- α -1-palmitoyl-2-[1- 14 C]linoleoyl phosphatidylcholine, and L- α -1-palmitoyl-2-[1- 14 C]arachidonoyl phosphatidylcholine were purchased from NEN Life Science Products. 1-*O*-(Z)-hexadec-1'-enyl-2-[9,10- 3 H]octadec-9'-enoyl-*sn*-glycero-3-phosphocholine was synthesized and purified as described in Han et al., *Anal. Biochem.* 200:119-24, 1992, which is herein incorporated by reference. The luciferase assay kit used was that of Promega. BEL was obtained from Sigma. Any other reagents were also obtained primarily from Sigma. Searches of EMBL and NCBI data bases were performed using the Basic Local Alignment Search Tool (BLAST) (NCBI). Alignments of all sequences were performed using the MultiAlign computer program, as described in Corpet, F., *Nucleic Acids Res.* 16:10881-90, 1988, which is herein incorporated by reference.

Expression of iPLA $_2\gamma$ in an intact eukaryotic system was evaluated using cultured *Spodoptera frugiperda* (Sf9) cells. Sf9 cells have been widely used to express recombinant proteins as a host cell in the baculovirus expression system. (See

e.g. Liu et al., *J. Immunol.* 156:3292-300, 1996). It is believed that the translational elements of Sf9 cells exhibit a high degree of homology with mammalian translational elements. (See e.g. Sakahira et al., *J. Biol. Chem.* 274:15740-4, 1999; Kimball et al., *J Biol. Chem.* 273:12841-5, 1998; Gu et al., *Biochim. Biophys. Acta* 1399:1-9, 1998).

5 Sf9 cells were grown and infected with wild-type or recombinant baculovirus containing human phospholipase A₂γ cDNA as described in detail in Wolf, M.J. and Gross, R.W., *J. Biol. Chem.* 271:30879-85, 1996, which is herein incorporated by reference. Culture of Sf9 cells involved, in brief, placing Sf9 cells in growth medium in 100-ml spinner flasks equipped with a magnetic spinner. The rotation speed of the
10 magnetic spinner was set at about 80 rpm, which is believed to be especially suitable for optimizing growth of Sf9 cells. The growth medium included Grace's medium supplemented with Yeastolate, Lactalbumin, 1% Antimycotic (all from Sigma), 10% fetal bovine serum and 1% Plutonic F-68 surfactant. Sf9 cells at a concentration of 1x10⁶ cells/ml were prepared in 50 ml of the growth medium and incubated at 27°C
15 for one (1) hour prior to infection with either wild-type baculovirus or recombinant baculovirus containing human phospholipase A₂γ cDNA. After 48 hours, cells were repelleted by centrifugation, resuspended in ice-cold phosphate-buffered saline, and repelleted. All subsequent operation were performed at 4°C. The supernatant was decanted, and the cell pellet was resuspended in 5 ml homogenization buffer (25 mM
20 imiazole, pH 8.0, 1 mM EGTA, 1mM dithiothreitol, 0.34 M sucrose, 20 MM transepoxy succinyl-L-leucylamido-(4-guanidino) butane, and 2μg/ml leupeptin. Cells were lysed at 0°C by sonication (twenty (20) one-second bursts utilizing a Vibra-cell sonicator at a 30% output) and centrifuged at 100,000 x g for one (1) hour. The supernatant containing predominantly cytosol was saved, and the membrane pellet
25 was washed with homogenization buffer and resuspended using a Teflon homogenizer in 6 ml of homogenization buffer. After a brief sonication (ten (10) one-second bursts), the mixture was subjected to recentrifugation at 100,000 x g for one (1) hour. After removal of the supernatant, the membrane pellet was resuspended in 1 ml homogenization buffer using a Teflon homogenizer and subsequently sonicated at 0°C
30 for five (5) one-second bursts.

For PCR, primer design and selection of appropriate restriction sites for constructing vectors proceeded according to the following. The free software program Amplify 1.2 was used to design nucleotides containing appropriate terminal restriction sites. In general, oligonucleotides are at least 20 nucleotides long, with approximately 50% GC/AT ratio, and do not have a T at the 3' end. The Amplify program predicts the efficiency of nucleotide priming, the presence of primer dimer pairing, if any, and the predicted sizes and approximate amounts of products obtained. The iPLA₂ used for designing primers has been deposited in the GenBank™ database (accession number AF263613).

For PCR amplification of iPLA₂ γ , the reagents (10x PCR buffer, Mg buffer, dNTPs, and Amplitaq) were obtained from PE Biosystems. Plugged pipet tips were used in all PCR procedures to minimize cross-contamination. PCR amplification occurred in the Perkin Elmer model 460 thermocycler. PCR reactions (50 μ l) were set up as follows:

15	10x PCR buffer	50 μ l
	Mg	3 μ l
	10 mM dNTO mix	1 μ l each
	sense primer	0.5 μ l of 0.08 μ M stock
	antisense primer	0.5 μ l of 0.08 μ M stock
20	DNA template	1 μ l of miniprep (from a 50 μ l miniprep stock)
	Sterile millipor water	35.75 μ l

The thermocycler was programmed to heat the above reaction mixture for five minutes at 94 °C followed by two minutes at 80 °C during which 0.25 μ l Amplitaq and one drop of oil were added prior to initiation of the cycling program.

For typical PCR analysis, a 30-cycle program was employed, with steps at 53 °C for 30 seconds, 72 °C for two minutes, and 94 °C for 30 seconds per cycle. IPLA₂γ was amplified utilizing oligonucleotides that flanked the predicted 5'- and 3'-coding region, M444 (5'-TTTTGTCGACATGTCTATTAATCTGACTGTAGATA-3') (SEQ ID NO:29) and M449 (5'-GCATACTCGAGTCACAATTTTGAAAAGAATGGAAGTCC-3')(SEQ ID NO:30), respectively to engineer appropriate restriction sites onto iPLA₂γ for subsequent cloning into SaII/SphI restriction sites of a pFASTBAC vector (Life Technologies, Inc.). PCR screening was performed using human skeletal muscle cDNA (0.5 ng), human heart Marathon cDNA (0.5 ng) and a human heart cDNA library (approximately 1x10⁹ plaque-forming units) as templates. To directly compare differences between the sequences reported herein and previously reported sequences, PCR amplification of sequence previously reported in a BAC genomic clone (Research Genetics) was used as a template, and PCR was performed with primers M452 (5'-GTACATACGGTGGACAAGCCTA-3')(SEQ ID NO:31) and M446 (5'-CATTCCTCTCCCTTTCCTGGATCCACATAGCC-3')(SEQ ID NO:32).

All PCR products were resolved by 1% agarose gel electrophoresis. Candidate bands visualized with ethidium bromide staining were extracted from the agarose gel using the QIAquick Gel extraction kit from Qiagen. The extracted DNA and cloning vector (pFASTBAC) were restriction digested with appropriate restriction enzymes (SaII/SphI for the above primers), resolved on a 1% agarose gel, extracted with the QIAquick Gel extraction kit, and then ligated. In a typical ligation 2 to 10 molar excess restriction digested PCR product was ligated overnight at 16 °C to restriction digested vector in a 20 µl reaction using T4 DNA ligase (Promega). For transformation, Stratagene XL2-Blue MRF ultracompetent cells (#200151) were used according to the manufacturer's protocol. Following bacterial transformation and growth of transformants, plasmids were purified using a QIAfilter plasmid kit from QIAGEN, and subjected to automated sequence analysis using either an ABI 373S or 377XL automated DNA sequencer (PE Biosystems).

Bacmids were prepared from the pFASTBAC plasmid constructs using the bac-to-Bac Baculovirus Expression System protocol from Life technologies, Inc. In brief, pFASTBAC plasmid constructs were used to transform DH10Bac competent cells resulting in a Tn7 site-specific transposition to produce a composite bacmid
 5 containing the cloned sequence of interest. This bacmid was used in Cellfectin-mediated transfection of Sf9 cells in 35 mm plates for 72 hours resulting in production of infectious recombinant baculovirus in the Sf9 cells. Recombinant baculovirus (1 ml) obtained was used to infect 50 ml of Sf9 cells in a 100 ml spinner for an additional 72 hours to provide a stock of amplified virus.

10 To prepare truncated iPLA₂γ constructs in pFASTBAC, PCR primers containing appropriate restriction sites for cloning were used. For example, the original full-length construct was used as a template in PCR with a sense primer annealing at nt 650-676 paired with reverse primer M458 to create a truncated iPLA₂γ (63kDapF) with a methionine start site corresponding to amino acid 221 in the full-
 15 length protein. Expression of this construct in the baculovirus system resulted in a 63 kDa recombinant protein. A second construct was created with a start site corresponding to methionine 122 to produce a 74kDa recombinant protein. Additional constructs were prepared to produce Nterminal truncated recombinant proteins of approximately 77, 70 and 66kDa. Segments of iPLA₂γ 5' sequence ranging
 20 between 15-663 nt were inserted 5' of the insert sequence in 63kDapF to identify potential sequences involved in translational regulation. These sequences were ligated as inframe 5' coding sequence or as 5' noncoding sequence to the 63kDapF construct. For recombinant protein produced in the Sf9 system as described herein, enzymatic activity was determined using the enzyme assay as described below in Example 6, and
 25 mass of each of these constructs was determined using Western analysis as described herein. Additionally, full-length truncated iPLA₂γ and truncated iPLA₂γ constructs were inserted 5' of full-length luciferase coding sequence which was cloned into pFASTBAC via NotI/XbaI restriction sites. Luciferase activity of recombinant protein produced in the Sf9 system was then measured using the Luciferase Assay
 30 System of Promega, as described in detail in Example 8 below. (Wood, K.V., (1991)

In: *Bioluminescence and Chemiluminescence: Current Status*, Stanley, P. and Kricka, L. eds., John Wiley and Sons, Chichester, NY, 543; and *Luciferase Assay System Technical Bulletin*, #TB101, Promega Corporation, 1996, which are herein incorporated by reference).

5 The unique ability of antibodies to recognize and specifically bind to target proteins provides an approach for treating an over expression of the protein. Thus, another aspect of the present invention provides for a method for preventing or treating diseases involving over expression of the iPLA₂γ protein by treatment of a patient with specific antibodies to the iPLA₂γ protein. Specific antibodies, either
10 polyclonal or monoclonal, to iPLA₂γ protein can be produced by any suitable method known in the art. For example, the iPLA₂γ protein, or an immunologically active fragment thereof, or an anti-idiotypic antibody, or fragment thereof can be administered to an animal to elicit the production of antibodies capable of recognizing and binding to the iPLA₂γ protein. Such antibodies can be from any class of
15 antibodies including, but not limited to IgG, IgA, IgM, IgD, and IgE or in the case of avian species, IgY and from any subclass of antibodies. Polyclonal antibodies can be prepared by immunizing rabbits or other animals by injecting antigen followed by subsequent boosts at appropriate intervals. The animals are bled and sera assayed against purified iPLA₂γ protein usually by ELISA or by bioassay based upon the
20 ability to block the activity of iPLA₂γ protein in cells. When using avian species, e.g. chicken, turkey and the like, the antibody can be isolated from the yolk of the egg. Monoclonal antibodies can be prepared after the method of Milstein and Kohler by fusing splenocytes from immunized mice with continuously replicating tumor cells such as myeloma or lymphoma cells. (Milstein and Kohler, *Nature* 256:495-497,
25 1975; Gutfre and Milstein, *METHODS IN ENZYMOLOGY: IMMUNOCHEMICAL TECHNIQUES* 73:1-46, Langone and Banatis eds., Academic Press, 1981 which are incorporated by reference). The hybridoma cells so formed are then cloned by limiting dilution methods and supernates assayed for antibody production by ELISA, RIA or bioassay.

More specifically, to generate and purify the anti-iPLA₂γ peptide polyclonal antibodies used in the work described herein, New Zealand white rabbits were immunized with the iPLA₂γ synthetic peptide CENIPLDESRNEKLDQ (SEQ ID NO:33). This peptide (G3, 4 mg), dissolved in minimal volume of dimethylsulfoxide, was conjugated to maleimide-activated keyhole limpet hemocyanin (KLH, 2 mg) in 200 μl of 83 mM sodium phosphate, 0.9 M NaCl, and 0.1 mM EDTA at 22 °C for two hours. After extensive dialysis against 83 mM sodium phosphate containing 0.9 mM NaCl, a 1:1 emulsion was made with the peptide-KLH conjugate and Freund's complete adjuvant. This emulsion was then injected subcutaneously into two anaesthetized New Zealand white rabbits. Two booster injections of a 1:1 emulsion of the peptide-KLH conjugate and Freund's incomplete adjuvant were given two and four weeks after the initial immunization. Serum was then collected for purification of the anti iPLA₂γ antibodies.

A peptide affinity column was generated by coupling the iPLA₂γ synthetic peptide to a thiopropyl-Sepharose (Amersham-Pharmacia) resin in the presence of 100 mM sodium citrate, pH 4.5. The resin-peptide suspension was mixed by inversion overnight at 22 °C. Coupling efficiency was monitored by the displacement of the 2-pyridyl sulfide groups as 2-thiopyridone through spectrophotometric monitoring at 343 nm. Before chromatography, the resin-peptide suspension was equilibrated with 10 mM Tris-HCl, pH 7.5 in an Econo-pac column (BioRad) by gravity flow. Serum containing the anti the iPLA₂γ antibodies was diluted 1:10 (v/v) with 10 mM Tris-HCl, pH 7.5 and then applied to the peptide affinity column by gravity flow. The diluted serum was then re-applied twice to the column to assure binding of the antibody to the column. After washing the column extensively with 10 mM Tris-HCl, pH 7.5 containing 500 mM NaCl, bound anti-iPLA₂γ antibody was eluted from the column with 100 mM glycine, pH 2.5 and the immediately neutralized with 100 mM Tris-HCl, pH 9.0. The antibody was then concentrated using Centricon-10 (Amicon) centrifugal filter devices and stored at 40 °C until use.

EXAMPLE 1

This example illustrates the approach used in identifying the full-length cDNA encoding phospholipase A₂γ.

Inspection of a sequence encoding a putative 40kDa phospholipase reported by the Human Genome Sequencing Project (BAC clone RG054D04; GenBankTM accession no. AC005058) demonstrated that the sequence did not begin with an initiator methionine codon. Thus, a TBLASTN data base search was undertaken to find expressed sequence tags (EST's) that might align with the 5'-end of the putative phospholipase sequence.

Six expressed sequence tags (EST's) identified in the TBLASTN search were mapped on the basis of their overlapping the 40kDa cDNA sequence or on the basis of their binding in close spatial proximity to the putative phospholipase sequence. Figure 8 is a schematic illustration showing this mapping. Amino acid numbering starting from the most 5' potential translation site is indicated by the scale at top, with a schematic representation of the previously identified putative 40-kDa phospholipase shown shaded below. The EST sequences that were used to identify a potential N-terminal initiator methionine and to map the full-length sequence of a novel phospholipase, calcium-independent phospholipase A₂γ (iPLA₂γ), are indicated by solid bars,. The accession number for each EST sequence is indicated above each solid bar. The arrows at bottom indicate the position and orientation of PCR primers M444 (5'-end) and M449 (3'-end) used to amplify a full-length 2.4-kb coding sequence for iPLA₂γ from human heart cDNA.

Specifically, EST clones vz36b01.ri Soares 2NbMT *Mus musculus* cDNA IMAGE:1328521 (accession no. AA915661) and *Rattus norvegicus* cDNA UI-R-C0-hp-c-06-0-UI (accession no. AA998901) were found to overlap with the BAC clone sequence, thereby adding an additional 360 nt sequence to the previously known 5' end of the putative phospholipase sequence. Four other EST clones found in the GenBankTM database (Stratagene *Homo sapiens* colon cDNA clone IMAGE:588479, accession no. AA143503; Stratagene *H. sapiens* cDNA clone IMAGE:647744, accession no. AA205528; normalized rat ovary, Bento soares *Rattus* sp. CDNA clone

ROVAA46, accession no. AA801084; and NCI_CGAP_GCB1 *H. sapiens* cDNA clone IMAGE:825005, accession no. AA504219) were found to be in close spatial proximity to the putative phospholipase sequence from the BAC clone. However, when aligned with the BAC clone sequence, the 3'-end of the EST AA504219

5 sequence and the 5'-end of EST AA998901 are separated by a 150-nt gap, as shown in Figure 8. Moreover, when the EST AA998901 sequence was back-translated through the gap and into EST AA504219, a continuous reading frame resulted. Thus, by overlapping known EST sequences and back-translating through the 150-nt gap, the inventors have succeeded in extending the putative phospholipase sequence
10 approximately 1.2 kb upstream from the predicted GenBank™ protein product.

The furthest upstream ATG codon that remained in frame with the newly identified full-length coding sequence was located 1210 nt upstream from the previously reported BAC clone sequence. Translation of the reported gene sequence further 5' from nt 122761 in BAC clone RG054D04 results in stop codons in all three
15 reading frames.

Based upon the predicted sequence obtained from this assembly of sequences in the database, primers were constructed to obtain and sequence the putative full-length phospholipase. For PCR, primer design and selection of appropriate restriction sites for constructing vectors proceeded according to the
20 following. The free software program Amplify 1.2 was used to design nucleotides containing appropriate terminal restriction sites. In general, oligonucleotides are at least 20 nucleotides long, with approximately 50% GC/AT ratio, and do not have a T at the 3' end. The Amplify program predicts the efficiency of nucleotide priming, the presence of primer dimer pairing, if any, and the predicted sizes and approximate
25 amounts of products obtained. The iPLA₂ used for designing primers has been deposited in the GenBank™ database (accession number AF263613).

PCR analysis used primers corresponding to the most 5' candidate initiator methionine and the known 3' stop codon (nt 79,673) in the gene sequence.

For typical PCR analysis, a 30-cycle program was employed with steps at 53 °C for 30 seconds, 72 °C for two minutes, and 94 °C for 30 seconds per cycle. iPLA₂γ was amplified utilizing oligonucleotides that flanked the predicted 5'- and 3'-coding region, M444 (5'-TTTGTCTGACATGTCTATTAATCTGACTGTAGATA-3') and M449 (5'-GCATACTCGAGTCACAATTTTGAAAAGAATGGAAGTCC-3'), respectively. PCR screening was performed using human skeletal muscle cDNA (0.5 ng), human heart Marathon cDNA (0.5 ng) and a human heart cDNA library (approximately 1x10⁹ plaque-forming units) as templates. To directly compare differences between the sequences reported herein and previously reported sequences, PCR amplification of sequence previously reported in BAC genomic clone RG054 D04 (Research Genetics) was used as a template, and PCR was performed with primers M452 (5'-GTACATACGGTGGACAAGCCTA-3')(SEQ ID NO:34) and M446 (5'-CATTCCTCTCCCTTTCACTGGATCCACATAGCC-3')(SEQ ID NO:35).

All PCR products were resolved by 1% agarose gel electrophoresis. Candidate bands were extracted from the agarose gel using the QIAquick Gel extraction kit from Qiagen followed by blunt end ligation into the pGEM-T-Vvector (Promega) by standard procedures. (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989, which is incorporated by reference). Following bacterial transformation and growth of transformants, plasmids were purified using a QIAfilter plasmid kit from CLONTECH, and subjected to automated sequence analysis using either an ABI 373S or 377XL automated DNA sequencer (PE Biosystems).

Figure 9 shows the results of PCR amplification of the novel human iPLA₂ sequence, iPLA₂γ. Specifically, PCR was performed using human heart cDNA (0.5 ng; *lane 1* in Figure 9), a cDNA library prepared from human heart (*lane 2*), human skeletal muscle (*lane 3*), and a blank control (*lane 4*), as templates as described in detail above. PCR primer M444 was positioned at the 5'-end, and primer M449 positioned at the 3'-end of the iPLA₂γ coding sequence, in 30 cycles of amplification

at 53°C for 30 seconds, 72°C for 2 minutes, and 94°C for 30 seconds. PCR products were analyzed on a 1% agarose gel and visualized using ethidium staining. Molecular size markers are shown at left in kb. PCR analysis gave rise to a single band, which was 2.4 kb in length, as indicated by the arrow.

5

EXAMPLE 2

This example illustrates the determination and characterization of the full length amino acid sequence of iPLA₂γ, based on the PCR product described in Example 1, Figure 9.

First, the PCR product was cloned and the sequence analyzed. More specifically, the PCR product was subcloned into pGEM-T Vector and sequenced in both directions. Based on amino acid residue 1 being the initiator methionine, the message encoded a 782-amino acid polypeptide with a calculated molecular weight of 88,476.9. Contained within the sequence were an ATP binding motif at amino acid residues 449-45, and a lipase consensus sequence at amino acid residues 481-485, as well as multiple cAMP phosphorylation sites, PKC phosphorylation sites, CK2 phosphorylation sites, and a microbody C-terminal targeting sequence as determined by a Prosite pattern search (Hofmann et al., *Nucleic Acids Res.* 27: 215-19, 1999; Bucher, P. and Bairoch, A. *ISMB-94 Proceedings: 2nd International Conference on Intelligent Systems for Molecular Biology, Menlo Park, CA*, pp. 53-61, AAAI Press, Menlo Park, CA, 1994, which are incorporated herein by reference).

Figure 10 is a schematic representation of the strategy utilized for sequencing the PCR product encoding iPLA₂γ. Size in nucleotides is indicated by the scale at top. Below the size scale is a schematic representation of the full-length iPLA₂γ coding sequence, indicating the location of the ATP binding consensus sequence at nt 1345-1362, and lipase consensus sequence at nt 1441-1445. The arrows represent the direction and length of the sequences obtained from individual sequencing reactions. At the bottom is a schematic enlargement of a region of

sequencing differences between the iPLA₂γ sequence as determined by the inventors, and the previously reported BAC clone sequence of a putative phospholipase.

As shown at the bottom of Figure 10, in the region between amino acid residues 351 and 358, the sequence analysis demonstrated ten putative differences between the iPLA₂γ sequence as determined by the inventors, and the previously reported GenBank™ sequence. However, three pieces of information substantiate the integrity of the of the sequence determined by the inventors. First, rat clone UI-R-C0-hp-c-06--0-UI (accession number AA998901) EST sequence agreed in its entirety with the sequence identified by the inventors. Second, the inventors' sequencing of the region of difference between their iPLA₂γ sequence and the previously reported GenBank™ sequence, in both directions from multiple different libraries, gave identical results. Third, sequencing of the original BAC clone in both directions produced identical results to those shown in Figure 10. Accordingly, it is believed that multiple errors are present in GenBank™ sequence RG054D04 in residues 351-358, and that the sequence shown in Figure 10 is correct.

To further analyze the deduced iPLA₂γ amino acid sequence shown above in Figure 10, a Kyte-Doolittle hydrophobicity analysis was performed on the sequence. (Kyte, J. and Doolittle, R.F., *J. Mol. Biol.* 157:105-132, 1982, which is herein incorporated by reference). Figure 11 shows a hydropathy plot of the deduced amino acid sequence of human iPLA₂γ. A window size of 17 amino acids was used. Negative values on the y-axis represent increasing hydrophobicity. Hydrophobicity analysis thus revealed that the putative iPLA₂γ had two major hydrophobic domains, a first at the extreme putative N-terminus and a second centered near the lipase consensus sequence, at Ser⁴⁸³.

To determine whether the iPLA₂γ PCR product represented the true 5'-end of the coding sequence, and to locate additional message sequence 5' of nt 122,761, 5'-RACE was performed using a reverse primer (M460) near the 5'-end of the 2.4 kb phospholipase A₂γ PCR product, and a sense primer (AP1) to the adapter sequence flanking the cDNA template.

More specifically, for 5'-RACE, a 45-cycle program with steps at 58 °C for 30 seconds, 72 °C for 2 minutes, and 94 °C for 30 seconds per cycle was employed. Human heart Marathon-ready cDNA was used as a template (0.5 ng) and primer AP1 (CLONTECH) was paired with M460 (5'-

5 GAAAACCTCTTTGTAGACTGATGTGGCTTATCCTCCAG-3')(SEQ ID NO:36) to amplify products. Products were analyzed by electrophoresis utilizing a 1% agarose gel and visualized by ethidium bromide staining. PCR products were excised from the gel, purified with a QIAquick gel extraction kit, and subcloned into pGEM-T vector (Promega) for sequencing and alignment with the iPLA₂γ sequence.

10 The full-length human iPLA₂γ message sequence as determined using 5'-RACE includes a putative transcription initiation site, 5'-untranslated region, coding sequence, and 3'-untranslated region that together form a 3.4kb mature message. Thus, this maneuver extended the 5' sequence from the putative initiator methionine residue an additional 225 base pairs upstream. Because the 225-base pair

15 sequence contained stop codons in all three reading frames, it is believed that no additional coding regions are present in the 225-base pair sequence. The most 5' ATG site that is in frame with the calcium-independent phospholipase A₂γ is located at nt 226. Additional in-frame ATG sites are located at nt 526, 589 and 886. Initiation of polypeptide synthesis at these potential methionine start sites would result in

20 polypeptides of 77, 74 and 63 kDa, respectively. At the 3'-end of the gene, a signal site for 3' poly(A) processing (AATAAA) was identified 757 nt 3' of the TGA stop signal. The actual cleavage site for poly(A) addition usually occurs 10-30 nt 3' of the poly(A) signal, and at this location, a CA is believed to be the preferred sequence immediately 5' to the cleavage site. Additionally, GU-rich or U-rich elements are also

25 typically found downstream of the poly(A) site. Since a CA dinucleotide occurs 35 nt 3' of the poly(A) signal sequence in iPLA₂γ, and highly U-rich sequences occur at nucleotides 3339-3343 and at 3373-3392, the inventors have deduced that the likely point of poly(A) addition occurs following nucleotide 3372. Accordingly, these results identify a putative transcription initiation site, 5'-untranslated region, coding

sequence, and 3'-untranslated region that together form a 3.4 kb mature message for iPLA₂γ.

To determine if the 3.4 kb message was the full-length or nearly full-length message, or if additional, as yet unidentified regions of the gene were transcribed to potentially serve a promoter function, Northern blot analysis of human iPLA₂γ mRNA was performed on multiple tissues.

For Northern blot analysis, full-length of iPLA₂γ amplified by PCR was prepared for use as a probe by radiolabeling with [³²P]dCTP for one (1) hour at 37 °C in the presence of Ready-to-Go labeling beads (Amersham Pharmacia) according to the instructions provided by the manufacturer. The radiolabeled probe was purified by gel filtration employing a 1-ml Sepharose G-25 spin column. For Northern analysis, and MTN blot (CLONTECH) containing 2 μg of poly(A)⁺ RNA/lane from human brain, heart, liver, lung, pancreas and placental tissue was prehybridized at 68 °C for 30 minutes in hybridization buffer, hybridized for one hour at 68 °C with radiolabeled iPLA₂γ (2 x 10⁶ cpm/ml), and washed in 2xSSC and 0.1% SDS twice for 30 minutes, followed by two washes with 0.1xSSC and 0.1% SDS for 40 minutes each at 50 °C as per the manufacturer's instructions. Hybridized sequences were identified by autoradiography for 16 hours.

Figure 12 shows the results of a Northern blot analysis of human iPLA₂γ mRNA. The Northern blot was human mRNA obtained from the multiple tissues, and the tissue distribution of iPLA₂γ mRNA thus examined by hybridization of the ³²P-labeled full-length iPLA₂γ with approximately 2 μg of human poly(A)⁺ mRNA, followed by autoradiography, as described above. *Lane 1* shows the results from heart, *lane 2* from brain, *lane 3* from placenta, *lane 4* lung, *lane 5* from liver, *lane 6* from skeletal muscle, *lane 7* from kidney, and *lane 8* from pancreas. The positions of RNA size markers are shown. Relative size in kb is indicated at left based on the mobility of the RNA standard ladder.

Northern blotting of human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas mRNA demonstrated that each of these tissues possessed a 3.4 kb message that tightly bound to the full-length ^{32}P -labeled probe. The largest amount of message was present in heart, followed by the placenta and skeletal muscle, with smaller amounts present in brain, kidney, pancreas, lung and liver. Within the limits of resolution of the Northern blotting technique, only a single band was identified. Thus, the results indicate that the message identified by the inventors and shown in Figure 12 is either full-length or nearly full-length. Multiple additional 5'-RACE reactions from multiple libraries did not identify any further sequence.

EXAMPLE 3

This example illustrates analysis of the complete genomic organization of the iPLA $_2\gamma$ gene. To determine the genomic organization of the full-length iPLA $_2\gamma$ gene, an examination was undertaken of sequence upstream from the putative start site of the BAC genomic clone (GenBank™ accession number AC005058).

Examination of the iPLA $_2\gamma$ sequence upstream from the putative transcription start site did not reveal the presence of either TATA box, nor CAAT box consensus sequences. The full-length message of iPLA $_2\gamma$ was aligned with BAC genomic clone RG054D04 sequence to determine the location of intron/exon boundaries. Two intron/exon boundaries were then identified within the 5'-untranslated region of the iPLA $_2\gamma$ sequence utilizing conventional AG/GT splicing rules.

Figure 13 is a schematic illustration of the human iPLA $_2\gamma$ gene, deduced by determining the locations of intron/exon boundaries. Intron/exon boundaries of the iPLA $_2\gamma$ gene are shown in scale (in kilobases). The eleven (11) exons of the gene are indicated as open boxes. Spaces between the exons represent the relative sizes of the ten (10) introns contained within the gene. Regions of the gene that correspond to the ATP binding, lipase and peroxisomal localization consensus sequences are indicated in exons 5, 6 and 11, respectively. The dashed box

enclosing exons 5-11 corresponds to previously identified exons in the BAC genomic clone (GenBank™ accession number AC005058). The boxes at the bottom indicate the nucleotide numbers corresponding to the original BAC genomic clone report, with the sizes of each exon in nucleotides and in amino acids shown within.

5 More specifically, exon 1 of the iPLA₂γ gene is 96 nt in length,
(nucleotides 133,299-133,394 of BAC genomic clone RG054D04) and is followed by
a 4.5-kb intron. Exon 2 is 46 nt in length, (nucleotides 128,746-128,791 of BAC
genomic clone RG054D04) and is followed by a 5.9-kb intron. The first candidate
ATG start site occurs 84 nt downstream from the start of exon 3 (nucleotides 121,692-
10 122,844 of BAC genomic clone RG054D04), which is 1151 nt in length and followed
by a 139-nt intron 3. Exon 4 is 139 nt in length, (nucleotides 121,411-121,522 of
BAC genomic clone RG054D04) and is followed by an 11.5-kb intron. Exon 5 begins
the coding sequence previously reported in the GenBank™ BAC clone report, as
indicated in Figure 13. Based on these findings, the iPLA₂γ gene on chromosome 7 is
15 54 kb in size and contains 11 exons, ranging in size from 46 to 1151 nucleotides.
Further, the results indicate that both the ATP binding motif (amino acid residues 449-
454), and the lipase consensus sequence (amino acid residues 481-485) of the iPLA₂γ
gene require splicing of adjacent exons to form complete functional sequences. This
splicing requirement distinguishes iPLA₂γ from the iPLA₂β, which contains its
20 functional ATP and lipase sites within a single exon.

Still further, these results show that the promoter region of the iPLA₂γ
gene contains neither a TATA box nor a CCAAT sequence within 400 nucleotides
flanking the putative transcription start site. However, Tfsearch analysis reveals other
promoter elements (e.g. several Sp1/GC boxes) that are located in the promoter
25 region, as well as several potential cap signals. One of the potential cap signals is
located immediately upstream of the predicted transcription initiation site (i.e. at
residues -6 to -13). TATA-less promoters are generally thought to typically respond
to a variety of stimuli, with a range of transcription regulatory responses including
differential expression during embryogenesis, tissue-specific distribution, and

regulation by either viral or pharmacologic stimuli. Many TATA-less genes are growth-regulated, so that low levels of gene expression are seen in non-growing cells but then expression up-regulated as cells proliferate. Thus, these results provide a foundation for studies in which the identified promoter region is fused to a reporter gene and subsequently dissected to determine important transcriptional regulatory elements by deletional mutagenesis.

To investigate the possible existence of iPLA₂γ splice variants including alternative locations of intron/exon boundaries, further analysis of the iPLA₂γ sequence upstream from the putative transcription start site of the BAC genomic clone was undertaken. Three alternative splice variants were discovered. Figure 2 is a schematic illustration of the three alternative splice variants of the iPLA₂γ gene, deduced in this further examination. A region of variation, including exons 1, 2 and 3 as identified above, is shown. Labeling indicates the size of corresponding protein in kDa, and length in nucleotides (nt). Open boxes are noncoding regions, shaded regions are putative coding regions, and stippled lines represent intron splicing. The three splice variants included a first, “Gamma 1”, that includes exon 1, exon 2 and a portion of exon 3 in noncoding region, with the start site in exon 3. A second, “Gamma 2” variant, includes exon 1 and a portion of exon 3 in noncoding region, but excludes exon 2 altogether. A third, “Gamma 3” variant, includes a new potential ATG start site in exon 1, and thus a portion of exon 1, all of exon 2 and a truncated exon 3 in coding region.

EXAMPLE 4

To determine the molecular weight of the protein(s) translated by the iPLA₂γ message, the 2.4 kb PCR product as described above was expressed in an *in vitro* reticulocyte lysate translation system, and the molecular weights of the protein products determined.

For *in vitro* translation, a full-length iPLA₂γ construct in pcDNA1.1 (1μg) was used in a coupled transcription/translation rabbit reticulocyte translation

lysate system (Promega) with RNA synthesis from the T7 promoter of pcDNA1.1 using T7 RNA polymerase and translation in the presence of 20 μ Ci of [35 S] methionine for 90 minutes according to the manufacturer's instructions. More specifically, the full-length iPLA $_2\gamma$ PCR product was cloned into pcDNA1.1 vector so that the T7 promoter region of pcDNA1.1 was upstream from the iPLA $_2\gamma$ sequence. For coupled *in vitro* transcription/translation, RNA was synthesized from 1 μ g of iPLA $_2\gamma$ -pcDNA1.1 in the presence of T7 RNA polymerase. Translation of RNA in the rabbit reticulocyte lysate system was performed in the presence of [35 S]methionine, and following translation, 5 μ l of labeled product was boiled for two minutes in SDS loading buffer. Translated, radiolabeled protein products were then resolved by SDS gel electrophoresis and visualized by autoradiography.

Figure 14 shows the resolution by gel electrophoresis and visualization by autoradiography of two radiolabeled protein products of iPLA $_2\gamma$ *in vitro* translation. . *In vitro* translated product corresponding to iPLA $_2\gamma$ is shown at left and indicated by " γ ", and a negative control indicated by "ctl" is shown at right. More specifically, Figure 14 shows the resolution a first product corresponding to a molecular mass of approximately 77 kDa and a second corresponding to a molecular mass of approximately 63 kDa. Sizes of molecular weight standards in kDa are indicated at left.

The first radiolabeled protein product of about 77 kDa corresponds to translation initiation at methionine residue 101 of the full-length 88 kDa. The second radiolabeled protein product of about 63 kDa corresponds to translation initiation at methionine residue 221 of the full-length iPLA $_2\gamma$.

EXAMPLE 5

To compare the results of *in vitro* translation as described in Example 4, with translation in an intact eukaryotic cell, the translation of 2.4 kb iPLA $_2\gamma$ in Sf9 cells was investigated.

This example thus illustrates the determination of the molecular weights of the two identified protein products of the iPLA₂γ gene as expressed in an intact eukaryotic cell, that the observed products correspond to results obtained in the *in vitro* translation as described in Example 4, and that iPLA₂γ protein products are present predominantly in the cell membrane.

Expression of iPLA₂γ in an intact eukaryotic system was evaluated using cultured *Spodoptera frugiperda* (Sf9) cells. Sf9 cells have been widely used to express recombinant proteins as a host cell in the baculovirus expression system. (See e.g. Liu et al., *J. Immunol.* 156:3292-300, 1996). It is believed that the translational elements of Sf9 cells exhibit a high degree of homology with mammalian translational elements. (See e.g. Sakahira et al., *J. Biol. Chem.* 274:15740-4, 1999; Kimball et al., *J Biol. Chem.* 273:12841-5, 1998; Gu et al., *Biochim. Biophys. Acta* 1399:1-9, 1998). Sf9 cells were grown and infected with wild-type or recombinant baculovirus containing human phospholipase A₂γ cDNA as described in detail in Wolf, M.J. and Gross, R.W., *J. Biol. Chem.* 271:30879-85, 1996, which is herein incorporated by reference. Culture of Sf9 cells involved, in brief, placing Sf9 cells in growth medium in 100-ml spinner flasks equipped with a magnetic spinner. The rotation speed of the magnetic spinner was set at about 80 rpm, which is believed to be especially suitable for optimizing growth of Sf9 cells. The growth medium included Grace's medium supplemented with Yeastolate, Lactalbumin, 1% Antimycotic (all from Sigma), 10% fetal bovine serum and 1% Plutonic F-68 surfactant. Sf9 cells at a concentration of 1x10⁶ cells/ml were prepared in 50 ml of the growth medium and incubated at 27°C for one (1) hour prior to infection with either wild-type baculovirus or recombinant baculovirus containing human phospholipase A₂γ cDNA. After 48 hours, cells were repelleted by centrifugation, resuspended in ice-cold phosphate-buffered saline, and repelleted. All subsequent operation were performed at 4°C. The supernatant was decanted, and the cell pellet was resuspended in 5 ml homogenization buffer (25 mM imiazole, pH 8.0, 1 mM EGTA, 1mM dithiothreitol, 0.34 M sucrose, 20 MM transepoxy succinyl-L-leucylamido-(4-guanidino) butane, and 2μg/ml leupeptin. Cells were lysed at 0°C by sonication (twenty (20) one-second bursts utilizing a Vibra-cell

sonicator at a 30% output) and centrifuged at 100,000 x g for one (1) hour. The supernatant containing predominantly cytosol was saved, and the membrane pellet was washed with homogenization buffer and resuspended using a Teflon homogenizer in 6 ml of homogenization buffer. After a brief sonication (ten (10) one-second
 5 bursts), the mixture was subjected to recentrifugation at 100,000 x g for one (1) hour. After removal of the supernatant, the membrane pellet was resuspended in 1 ml homogenization buffer using a Teflon homogenizer and subsequently sonicated at 0°C for five (5) one-second bursts.

The 2.4-kb message encoding iPLA₂γ as identified was inserted into
 10 the Sf9 cell vector pFASTBAC. Spinner cultures of Sf9 cells were infected with either the wild-type pFASTBAC (“Ctl”) or pFASTBAC containing the 2.4-kb message (“γ”). At 48 hours post-infection, the cells were collected and cytosolic (“Cyto”) and membrane (“Memb”) fractions prepared as described above.

Sf9 cell cytosol and membrane fractions were loaded on a 10%
 15 polyacrylamide gel, resolved by SDS polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, by electroelution in 10 mM CAPS, pH 11, containing 10% methanol. Dry powdered milk (5% w/v) in 20 mM Tris-HCl, pH 7.4, containing 137 mM NaCl and 0.1% Tween 20 was used to block non-specific binding sites before incubation with the primary antibody, immunoaffinity-purified antibody
 20 directed against iPLA₂γ, prepared as described below. Secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate) was incubated with the blot for 1 hour and immunoreactive bands were visualized by ECL.

Immunoaffinity-purified polyclonal antibody directed against iPLA₂γ was prepared by immunizing rabbits with the iPLA₂γ synthetic peptide
 25 CENIPLDESRNEKLDQ (SEQ ID NO:37). This peptide (G3, 4 mg), dissolved in minimal volume of dimethylsulfoxide, was conjugated to maleimide-activated keyhole limpet hemocyanin (KLH, 2 mg) in 200 μl of 83 mM sodium phosphate, 0.9 M NaCl, and 0.1 mM EDTA at 22 °C for two hours. After extensive dialysis against 83 mM sodium phosphate containing 0.9 mM NaCl, a 1:1 emulsion was made with

the peptide-KLH conjugate and Freund's complete adjuvant. This emulsion was then injected subcutaneously into two anaesthetized New Zealand white rabbits. Two booster injections of a 1:1 emulsion of the peptide-KLH conjugate and Freund's incomplete adjuvant were given two and four weeks after the initial immunization.

5 Serum was then A peptide affinity column was generated by coupling the iPLA₂γ synthetic peptide to a thiopropyl-Sepharose (Amersham-Pharmacia) resin in the presence of 100 mM sodium citrate, pH 4.5. The resin-peptide suspension was mixed by inversion overnight at 22 °C. Coupling efficiency was monitored by the displacement of the 2-pyridyl sulfide groups as 2-thiopyridone through
10 spectrophotometric monitoring at 343 nm. Before chromatography, the resin-peptide suspension was equilibrated with 10 mM Tris-HCl, pH 7.5 in an Econo-pac column (BioRad) by gravity flow. Serum containing the anti the iPLA₂γ antibodies was diluted 1:10 (v/v) with 10 mM Tris-HCl, pH 7.5 and then applied to the peptide affinity column by gravity flow. The diluted serum was then re-applied twice to the
15 column to assure binding of the antibody to the column. After washing the column extensively with 10 mM Tris-HCl, pH 7.5 containing 500 mM NaCl, bound anti-iPLA₂γ antibody was eluted from the column with 100 mM glycine, pH 2.5 and the immediately neutralized with 100 mM Tris-HCl, pH 9.0. The antibody was then concentrated using Centricon-10 (Amicon) centrifugal filter devices and stored at 40
20 °C until use.

Figure 15 shows the results of Western analysis of iPLA₂γ expression in Sf9 cells, and confirm in an intact eukaryotic system the identification of translation initiation sites at methionine residues 101 and 221 as identified in the *in vitro* system as described in Example 4. The results as shown in Figure 15 are typical of three
25 independent experiments. Specifically, Western analysis of membrane fractions from Sf9 cells infected with vector harboring iPLA₂γ ("γ Memb") demonstrated the presence of two major bands corresponding to molecular masses of 77 and 63 kDa. However, no such products were present in membrane fractions from Sf9 cultures infected with wild-type virus ("Ctl Memb"). In contrast, cytosolic fractions from
30 control or iPLA₂γ-transfected Sf9 cells did not contain any detectable immunoreactive

protein. Collectively, these results support the existence of two translation initiation start sites at methionine residues 101 and 221.

EXAMPLE 6

To determine whether iPLA₂γ translation products as described in Example 5 are biologically active, a phospholipase enzyme activity analysis was undertaken of Sf9 cells infected with recombinant baculovirus encoding iPLA₂γ. This example demonstrates that iPLA₂γ translation products are biologically active and more specifically, are catalysts of cleavage of the *sn*-2-fatty acid of choline glycerophospholipids

Briefly, Sf9 cells were infected with either wild-type baculovirus (□) or baculovirus encoding human iPLA₂γ (●), as described in Example 5. Cytosolic and membrane fractions of the infected Sf9 cells were prepared as described above. Phospholipase A₂ activity was assessed as follows. Membrane fractions from control cells or cells expressing iPLA₂γ were incubated with 40 μM L-α-1-palmitoyl-2-[1-¹⁴C]oleoyl phosphatidylcholine in 100mM Tris-Hac, pH 8.0, containing 1 mM EGTA at 37 °C. Aliquots of the reaction were removed at the indicated times, and the amount of [1-¹⁴C]oleic acid released was quantified as described above. Specifically, the release of *sn*-2-fatty acid from 1-hexadecanoyl-2-[1-¹⁴C]octadec-9'-enoyl-*sn*-glycero-3-phosphocholine was determined as a function of time.

Figure 16 is a graph showing an initial rate analysis of phospholipase activity in Sf9 cells infected with recombinant baculovirus encoding iPLA₂γ. The results shown in Figure 16 are representative of three independent experiments.

Membrane fractions from cells infected with vector harboring the iPLA₂γ insert released fatty acid from the *sn*-2-position of phosphatidylcholine approximately 10-fold faster than from membrane fractions prepared control cells infected with wild-type vector. Liberation of *sn*-2-radiolabeled fatty acid was nearly

linear for 2 minutes at a velocity of approximately 0.3 nmol/mg-min. Phospholipase A₂ activity was entirely calcium independent in the range of 0-10 mM and demonstrated a pH optimum at pH 8.0 (not shown), which is at or near physiologic pH.. In contrast, phospholipase A₂ activities in cytosolic fractions of cells infected with wild-type vector and from cells infected with vector harboring the iPLA₂γ insert were found to be similar (not shown).

To determine the substrate selectivity of the phospholipase A₂ activity demonstrated in Sf9 cells infected recombinant baculovirus encoding iPLA₂γ, multiple alternative phospholipase A₂ substrates were tested. Membrane fractions from both groups of cells were incubated in 100 mM Tris-Hac, pH 8.0, containing 1 mM EGTA for 2 minutes at 37 °C in the presence of either 40 μM L-α-1-dipalmitoyl-2-[1-¹⁴C]phosphatidylcholine ("PPPC"), L-α-1-palmitoyl-2-[1-¹⁴C]oleoyl phosphatidylcholine ("POPC"), L-α-1-palmitoyl-2-[1-¹⁴C]linoleoyl phosphatidylcholine ("PLPC"), or L-α-1-palmitoyl-2-[1-¹⁴C]arachidonyl phosphatidylcholine ("PAPC"). Reactions were terminated by butanol extraction, and radiolabeled fatty acids were resolved by TLC and quantitated by scintillation spectrometry.

Figure 17 is a substrate selectivity profile of iPLA₂γ phospholipase A₂ activity in Sf9 cells infected recombinant baculovirus encoding iPLA₂γ. Panel A shows the results of phospholipase A₂ activity analysis from Sf9 cells infected with either wild-type baculovirus (stippled bars) or recombinant baculovirus encoding iPLA₂γ (solid bars). The results are representative of three independent experiments. Thus, incubation of membrane fractions containing iPLA₂γ with phospholipids containing three distinct types of unsaturated fatty acids at the *sn*-2-position (oleic, linoleic, and arachidonic acids) gave similar specific activities that were each 6-10 times greater than activities manifest in membrane preparations derived from cells infected with wild-type vector

Figure 17, Panel B shows the results of further phospholipase A₂ activity analyses undertaken to identify the initial site of hydrolysis of phospholipids

by iPLA₂γ. Membranes derived from Sf9 infected with either wild-type baculovirus (stippled bars) or recombinant baculovirus encoding iPLA₂γ (solid bars) were incubated with either 40 μM L-α-1-palmitoyl-2-[1-¹⁴C]oleoyl phosphatidylcholine ("POPC"), or 40 μM synthetic radiolabeled 1-*O*-(*Z*)-hexadec-1'-enyl-2-[9,10-³H]octadec-9'-enoyl-*sn*-glycero-3-phosphocholine ("plasmeyl-PC"), under incubation conditions as described for Panel A. Plasmeyl choline has a relatively enzymatically resistant vinyl ether linkage at *sn*-1.

Panel B of Figure 17 shows that the rate of hydrolysis using plasmeyl choline was similar to that using *sn*-2-[1-¹⁴C]phosphatidylcholines, thus supporting the conclusion that hydrolysis of substrate by iPLA₂γ occurs predominantly at the *sn*-2-position. Therefore, iPLA₂γ gene products are catalysts of cleavage of the *sn*-2-fatty acid of choline glycerophospholipids.

EXAMPLE 7

To establish iPLA₂γ as a member of the calcium-independent phospholipase A₂ family, the inhibition of the phospholipase A₂ activity of iPLA₂γ by (E)-6-(bromomethylene)-3-(1-napthalenyl)-2H-tetrahydropyran-2-one (BEL) was studied.

(E)-6-(bromomethylene)-3-(1-napthalenyl)-2H-tetrahydropyran-2-one (BEL) has previously been shown to be a powerful and irreversible mechanism-based inhibitor of myocardial cytosolic and membrane-associated iPLA₂, with nearly complete inhibition of myocardial cytosolic membrane-associated iPLA₂ at concentrations of approximately 2-5 μM. (Hazen et al., *J. Biol. Chem.* 266:7227-32, 1991; Zupan et al., *J. Med. Chem.* 36: 95-100, 1991; Gross et al., *Biochemistry* 32:327-36, 1993; Ramanadham et al., *Biochemistry* 32:337-46, 1993, which are herein incorporated by reference).

Briefly, Sf9 cells were infected with either wild-type baculovirus (□) or baculovirus encoding human iPLA₂γ (●), as described in Example 5. Membrane fractions of the infected Sf9 cells were prepared as described above, and then

incubated for three minutes at room temperature in 100 mM Tris-HAc, pH 8.0, containing 1mM EGTA, with varying concentrations of BEL, or ethanol vehicle, as indicated along the x-axis. L- α -1-palmitoyl-2-[1- 14 C]oleoyl phosphatidylcholine (40 μ M final concentration) in ethanol was then added to each reaction, followed by

5 incubation at 37 °C for two minutes. Released [1- 14 C]oleic acid was quantitaed as described above.

Figure 18 is a graph showing inhibition of the phospholipase A₂ activity of iPLA₂ γ by (E)-6-(bromomethylene)-3-(1-napthalenyl)-2H-tetrahydropyran-2-one (BEL). The results shown in Figure 18 were representative of three

10 independent experiments.

As shown in Figure 18, preincubation of membranes harboring iPLA₂ γ with 5 μ M BEL for three minutes prior to the addition of substrate resulted in the inhibition of approximately 70% of iPLA₂ γ phospholipase A₂ activity. The IC₅₀ for BEL inhibition of iPLA₂ γ was approximately 3 μ M. Thus, the results establish that

15 BEL is an effective inhibitor of iPLA₂ γ .

Previous work has characterized iPLA₂ activity in certain tissues, as well as a calcium-*facilitated* phospholipase A₂ γ . Specifically, prior studies have identified iPLA₂ activity in cytosolic and membrane fractions of canine and human myocardium. (Wolf, R.A. and Gross, R.W., *J. Biol. Chem.* 260:7295-303, 1984;

20 Hazen, S.L. and Gross, R.W., *Circ. Res.* 70:486-95, 1992, which are herein incorporated by reference). Additional studies have demonstrated an increase in membrane-associated, BEL-inhibitable iPLA₂ activity during myocardial ischemia or hypoxia. (Hazen et al., *J. Biol. Chem.* 266:5629-33, 1991; Ford et al., *J. Clin. Inv.* 88:331-35, 1991; McHowat J. and Creer, M.H., *Lipids* 33:1203-12, 1998; McHowat

25 et al., *Am. J. Physiol.* 274:C1727-37, 1998, which are herein incorporated by reference). It has also been proposed that increased myocardial iPLA₂ activity during ischemia contributes to ventricular arrhythmias and hemodynamic dysfunction through the production of lysophospholipids and arachidonic acid, each of which has potent electrophysiological effects. (Wolf, R.A and Gross, R.W., *supra*; Gross, *Trends*

Cardiovasc. Med. 26:115-21, 1992; and Gross, *Biochem. Soc. Trans.* 26:345-49, 1984, which are herein incorporated by reference).

A calcium-facilitated phospholipase A₂γ shares homology with the catalytic domain of calcium-facilitated phospholipase A₂α and has been cloned and expressed. Northern analysis has demonstrated the presence of calcium-facilitated phospholipase A₂γ message in skeletal and heart muscle, and cloning and expression of calcium-facilitated phospholipase A₂γ in Sf9 cells has confirmed the membrane localization and calcium-independence of calcium-facilitated phospholipase A₂γ. (Underwood et al., *J. Biol. Chem.* 273:21926-32, 1998 which is herein incorporated by reference). However, calcium-facilitated phospholipase A₂γ is not inhibited by BEL at <50 μM [BEL] (C.M. Jenkins, D.J. Mancuso and R.W. Gross, manuscript in preparation) and is therefore clearly distinguishable from iPLA₂γ, which is subject to potent inhibition by BEL as shown in Figure 19. In contrast, the large majority of iPLA₂ activity from ischemic hearts or hypoxic myocytes in culture is membrane-associated and exquisitely sensitive to BEL inhibition. Accordingly, the inventors herein have succeeded in identifying iPLA₂γ as a likely polypeptide for catalyzing the increase in ischemia-induced iPLA₂ activity. Further, the results suggest that the peroxisomal compartment is a source of regulatory and potentially arrhythmogenic eicosanoid metabolites and lysophospholipids.

EXAMPLE 8

To determine whether iPLA₂γ expression is subject to translational control, multiple iPLA₂γ truncation mutants were prepared and their expression in Sf9 cells studied.

To prepare truncated iPLA₂γ constructs in pFASTBAC, PCR primers containing appropriate restriction sites for cloning were used, and performed as described above. For example, the original full-length 88 kDa construct was used as a template in PCR with a sense primer annealing at nt 650-676 paired with reverse primer M458 (5'-GCATAGCATGCTCACAATTTTGAAAAGAATGCAAGTCC-3')

(SEQ ID NO:38) to create a truncated iPLA₂γ (63kDapF) with a methionine start site corresponding to amino acid 221 in the full-length protein. Expression of this construct in the baculovirus system as described above resulted in a 63 kDa recombinant protein. A second construct was created with a start site corresponding to methionine 122 to produce a 74kDa recombinant protein. Additional constructs were also prepared as described above to produce N-terminal truncated recombinant proteins of approximately 77, 70 and 66kDa that were used in additional studies of translational inhibition as described below in Example 9 . Further, the same methods were used to for segments of iPLA₂γ 5' sequence ranging between 15-663 nt which were inserted 5' of the insert sequence in 63kDapF to identify potential sequences involved in translational regulation, as described below in Example 10.

The expression of the 63 kDa and the 74 kDa truncation mutants were studied in Sf9 cells using the expression system as described above. Cytosolic and membrane fractions were obtained as described above, and a Western analysis performed on the polypeptide products products of the 63 kDa and 74 kDa truncation mutants of iPLA₂γ, as expressed in cytosolic and membrane fractions prepared from Sf9 cells.

Figure 19 shows the results of the Western analysis. The wild-type 88 kDa protein is indicated by iPLA₂¹γ. Both cytosolic and membrane fractions (“63 kDa iPLA₂γ Cyto” and “63 kDa iPLA₂γ Memb”, respectively) from Sf9 cells infected with vector harboring truncation mutants coding the 63 kDa product of iPLA₂γ, demonstrated the presence of major bands corresponding to molecular mass of 63 kDa, though to a lesser extent in the cytosolic fraction. However, analysis of both cytosolic and membrane fractions (“74 kDa iPLA₂γ Cyto” and “74 kDa iPLA₂γ Memb”, respectively) from Sf9 cells infected with vector harboring truncation mutants coding the 74 kDa product of iPLA₂γ, demonstrated greatly decreased amounts of product in comparison to that expressed using the 63 kDa truncation mutant. Similarly, analysis of both cytosolic and membrane fractions (“iPLA₂¹γ Cyto” and “iPLA₂¹γ Memb”, respectively) from Sf9 cells infected with vector harboring

truncation mutants coding the iPLA₂¹ γ (i.e. wild-type 88 kDa protein), demonstrated little or no expressed product in comparison to that expressed using the 63 kDa truncation mutant. Collectively these results support the existence of a sequence or sequences involved in translation inhibition. Further, such sequences can be localized to the stretch of sequence representing the difference between the 63 kDa truncation mutant and the 74 kDa truncation mutant.

EXAMPLE 9

To more precisely localize the sequence involved in translational inhibition of iPLA₂ γ multiple constructs were prepared according to the methods described above.

Briefly, each construct tested included the 63 kDa truncation mutant as described above, plus one of the truncation mutants. Specifically then the first construct included the 63 kDa truncation mutant plus the 63 kDa truncation mutant, the second construct included the 63 kDa truncation mutant plus the 66 kDa truncation mutant, the third construct included the 63 kDa truncation mutant plus the 70 kDa truncation mutant, the fourth construct included the 63 kDa truncation mutant plus the 74 kDa truncation mutant, the fifth construct included the 63 kDa truncation mutant plus the 77 kDa truncation mutant, and the sixth construct included the 63 kDa truncation mutant plus the full-length 88 kDa truncation mutant, all prepared as described above.

Expression of each construct in the baculovirus system was then studied in Sf9 cells, using the expression and Western analysis methods as described above. Figure 20 is a schematic illustration of the results of Western analysis of polypeptide products of each construct as expressed in Sf9 cells. Western analysis of both cytosolic and membrane fractions from Sf9 cells infected with vector harboring truncation mutants coding the 63 kDa + 63 kDa product of iPLA₂ γ , demonstrated the expression of an immunoreactive band at 63 kDa. Similarly, Western analysis of both cytosolic and membrane fractions from Sf9 cells infected with vector harboring

truncation mutants coding the 63 kDa + 66 kDa product of iPLA₂γ, and those infected with vector harboring truncation mutants coding the 63 kDa + 70 kDa product, all demonstrated the expression of an immunoreactive band at 63 kDa. However, analysis of both cytosolic and membrane fractions from Sf9 cells infected with vector

5 harboring truncation mutants coding the 63 kDa + 74 kDa product of iPLA₂γ, demonstrated decreased immunoreactivity relative to those including the 63 kDa, 66 kDa or 70 kDa truncation mutants. Further, no immunoreactive band was detectable from cells containing the 77 kDa truncation mutant or the 88 kDa wild-type sequence. Thus, these results indicate translational inhibition by a sequence contained within the

10 77 kDa truncation mutant but not present in the 66 kDa or 70 kDa truncation mutants..

EXAMPLE 10

To further identify and locate the sequences involved in translational inhibition of iPLA₂γ, the effect on translation of several iPLA₂γ 23-mer sequences 5' of the 63 kDa sequence was studied.

15 Briefly, sequences from iPLA₂γ were inserted by ligation of 15-23-mer annealed phosphorylated oligonucleotide pairs 5' of full-length luciferase coding sequence which was cloned into pFASTBAC via NotI/XbaI restriction sites. Luciferase activity of recombinant protein produced in the Sf9 system was then measured using the Luciferase Assay System of Promega. (Wood, K.V., (1991) In:

20 *Bioluminescence and Chemiluminescence: Current Status*, Stanley, P. and Kricka, L. eds., John Wiley and Sons, Chichester, NY, 543; and *Luciferase Assay System Technical Bulletin*, #TB101, Promega Corporation, 1996, which are herein incorporated by reference). Figure 21 is a schematic diagram showing ligation of 23-mer iPLA₂γ sequence to luciferase coding sequence and cloned into pFASTBAC.

25 Figure 22 is a schematic representation of the location of the 23-mer sequences studied. Schematic representations of the the 74 kDa protein product of iPLA₂γ, as well as the 70kDa sequence, are shown at the top for comparison. "1/2", "3/4", "5/6", and "7/8" each indicate successive 23-mer DNA sequences taken from

coding region representing the difference between the sequences for a 70 kDa iPLA₂γ protein and the 74 kDa protein. The numbers 1-8 indicate the primers that were used to amplify each 23-mer piece. Constructs were prepared by fusing luciferase coding sequence to a one of the four alternative 23mer sequences from iPLA₂γ, and to the

5 baculovirus promoter. Four alternative constructs, each containing one of the four different 23mer sequences from the 74 kDa iPLA₂γ truncation mutant, were prepared and then expressed in Sf9 cells as described above. After 48 hours allowed for expression, cell extracts were prepared and then tested for level of expression using the luciferase assay system as described in detail above.

10 Figure 23 shows the phosphorylated oligo pairs for sequence between nucleotide 364-455 that is involved in translation inhibition of iPLA₂γ in the luciferase expression system, thus identifying four pairs, “1/2”, “3/4”, “5/6”, and “7/8” which each indicate successive 23-mer DNA sequences taken from coding region representing the difference between the sequences for a 70 kDa iPLA₂γ protein and

15 the 74 kDa protein.

Figure 24 is a bar graph showing luciferase assay results of the expression of three of the four different iPLA₂γ constructs. The graph plots relative light units (RLU) emitted for each of three constructs containing a different 23mer sequence from iPLA₂γ 74 kDa truncation mutant, as described above. The first

20 construct contained the 23-mer sequence identified as “1/2” on the *x*-axis, the second construct, contained the 23-mer sequence identified as “5/6” on the *x*-axis, and the third construct contained the 23-mer identified as “7/8” on the *x*-axis. The 3/4 sequence did not amplify. The results, including little or no expression of the construct containing the 1/2 sequence, thus show that at least a portion of 1/2 is

25 involved in translational inhibition of iPLA₂γ. In contrast, the construct containing 5/6 resulted in a relatively high level of expression of iPLA₂γ, showing 5/6 is unlikely to be involved in translational inhibition. The results for the third construct containing 7/8 showed an intermediate level of expression that was not significantly different from the 5/6 construct. Thus, 7/8 is not likely to be involved in translational

30 inhibition of iPLA₂γ. Thus, these results show that iPLA₂γ is subject to translational

inhibition by the 1/2 23-mer piece taken from coding region representing the difference between the sequences for a 70 kDa iPLA₂γ protein and the 74 kDa protein.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended to summarize the assertions made by their authors and no admission is made as to the accuracy or pertinency of the cited references or that any reference is material to patentability.

While the invention has been described in terms of various specific embodiments in the examples, those skilled in the art will recognize that the invention can be practiced with modification within the spirit and scope of the claims which follow.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising a polynucleotide encoding a phospholipase A₂ γ polypeptide.
2. An isolated nucleic acid molecule according to claim 1, wherein said phospholipase A₂ γ polypeptide catalyzes cleavage of fatty acids from the *sn*-2-position of phospholipids.
3. An isolated nucleic acid molecule according to claim 2 wherein said polynucleotide encodes a sequence as set forth in SEQ ID NO:1 or SEQ ID NO:2.
4. A vector comprising a nucleic acid molecule according to claim 1.
5. A cell transformed or transfected with a vector according to claim 4.
6. An isolated nucleic acid molecule comprising a fragment of a polynucleotide encoding a phospholipase A₂ γ wherein said fragment specifically hybridizes with a sequence as set forth in SEQ ID NO:5 or SEQ ID NO:6.
7. An isolated nucleic acid comprising a polynucleotide having at least about 90% identity with SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:6.
8. An isolated nucleic acid according to claim 7 comprising SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:6.
9. An antisense sequence which specifically hybridizes to SEQ ID NO:2, or SEQ ID NO:3 .
10. An isolated polypeptide comprising a phospholipase A₂ γ .
11. An isolated polypeptide according to claim 10 which catalyzes cleavage of fatty acids from the *sn*-2-position of phospholipids.
12. An isolated polypeptide according to claim 11 which has at least 90% identity with SEQ ID NO:1 or SEQ ID NO:2.
13. An isolated polypeptide according to claim 12 comprising SEQ ID NO:1 or SEQ ID NO:2.
14. An isolated polypeptide according to claim 12 which is a conservatively substituted variant of SEQ ID NO:1 or SEQ ID NO:2.
15. An antibody capable of binding to a phospholipase A₂ γ according to claim 10.

16. A method of treating inflammation in a patient, said method comprising decreasing calcium-independent phospholipase A₂γ activity in the patient.

17. A method according to claim 16 wherein the patient suffers from Alzheimer's disease, myocardial ischemia, or myocardial infarction.

5 18. A method according to claim 17 comprising administering to the patient a phospholipase A₂γ translational repressor molecule.

19. A method according to claim 17 comprising administering to the patient an antisense sequence which specifically hybridizes to SEQ ID NO:3 or SEQ ID NO:4 .

10 20. A method of increasing fatty acid utilization in a patient in need thereof, said method comprising increasing iPLA₂γ activity in the patient.

21. A method according to claim 20 wherein the patient suffers from diabetes or obesity.

22. A method according to claim 21 comprising administering to the patient a substance which blocks translational repression of iPLA₂γ expression.

15 23. A method according to claim 21 comprising administering to the patient an iPLA₂γ polypeptide as set forth in SEQ ID NO:1, SEQ ID NO:2 or a conservatively substituted variant thereof or administering a polynucleotide encoding said iPLA₂γ polypeptide.

20 24. A method for measuring activity of a phospholipase A₂γ polypeptide of cells in a biological sample, said method comprising introducing into the sample a phospholipid substrate for cleavage of fatty acids by the phospholipase A₂γ , wherein the phospholipase A₂γ cleaves fatty acid from the *sn*-2-position of the phospholipid substrate and measuring cleavage of the phospholipid substrate, wherein measuring cleavage of the phospholipid substrate comprises quantifying the release of fatty acid
25 from the substrate.

25. An assay method for identifying substances which modulate iPLA₂γ expression in a cell, said method comprising contacting a candidate substance with cells comprising a promoter sequence operably linked to an iPLA₂γ repressor binding site and a reporter gene and measuring expression of the reporter gene.

30 26. A method according to claim 25 wherein said repressor binding site comprises SEQ ID NO:7.

27. A method according to claim 26 wherein said reporter gene encodes an enzyme capable of being detected by a colorimetric, fluorimetric or luminometric assay.

28. A method according to claim 27 wherein said reporter gene encodes a luciferase.

29. A method according to claim 26 wherein the promoter sequence is a baculovirus promoter sequence.

30. A method according to claim 26 wherein the cells are Sf9 cells.

31. A genetically engineered cell capable of identifying substances which modulate iPLA_{2γ} expression in a cell, said cells comprising a promoter operably linked to an iPLA_{2γ} repressor binding site and a reporter gene.

32. A genetically engineered cell according to claim 31 wherein said repressor binding site comprises SEQ ID NO:7.

33. A genetically engineered cell according to claim 32 wherein said reporter gene encodes an enzyme capable of being detected by a colorimetric, fluorimetric or luminometric assay.

34. A genetically engineered cell according to claim 33 wherein said reporter gene encodes a luciferase.

35. A genetically engineered cell according to claim 32 wherein the promoter is a baculovirus promoter.

36. A genetically engineered cell according to claim 32 wherein the cells are Sf9 cells.

37. A method for identifying a substance which modulates iPLA_{2γ} expression, the method comprising: (1) contacting a candidate substance with a repressor binding site and detecting binding to said site, or (2) contacting a candidate substance with cells capable of expressing iPLA_{2γ} or a fragment thereof and measuring the expression of iPLA_{2γ} or fragment thereof by the cells, wherein a level of expression greater or less than that in absence of the substance indicates activity in modulating iPLA_{2γ} expression.

CALCIUM INDEPENDENT PHOSPHOLIPASE $A_{2\gamma}$
AND METHODS THEREFOR

ABSTRACT OF THE DISCLOSURE

A novel human phospholipase, iPLA $_2\gamma$ (iPL $_2\gamma$), is described. The complete genomic organization, complete nucleic acid sequence, and amino acid sequence have been identified. The *sn*-2-lipase activity of iPL $_2\gamma$ is also described. In addition, methods for detecting and monitoring patient levels of iPL $_2\gamma$ are described,
5 as well as methods for treating disease conditions by modulating the activity of iPL $_2\gamma$.

FIGURE 1 (SHEET 1)

```

1  TGAAGCTCAGCTGATGCAAGCGCGTTGGAGTGACGTCATTGCCGGGAACGACGAGTC  60
61  GCCGCTGCAGCCCTAGTACTGCGGCTGCATCCCGATTGCTCTCTCCTCCAAGTCTAC  120
121 ATGATTACCTGAAGTTTAAATAGTAAGACCATGAATTAAGCATTTCTTAAATGAAGCGT  180
181 TCAAGAAGTGAGAGAATGTCAATAGAAATAAATGATTTTAAAGTTATGCTATAATCTG  240
      M S I N L
241 ACTGTAGATATATATATTACCTCCTTAGTAATGCAAGAAGTGTGTTGGGAACGAGAGA  300
      T V D I Y I Y L L S N A R S V C G K Q R
301 AGCAAGCAACTGTATTTCTGTGTTCTCACCTAAGCATTACTGGAGATAAGCCACATCAGT  360
      S K Q L Y F L F S P K H Y W R I S H I S
361 CTACAAGAGGTTTCATACAAACATAATAAGATGTAATGACCATAAAGTGAAGCACAT  420
      L Q R G F H T N I I R C K W T K S E A H
421 TCTTGCAAGTAAAGCACTGTACTCTCCAAAGCAACCATGTTTACATATTGGGATTTTGA  480
      S C S K H C Y S P S N H G L H I G I L K
481 CTTAGCACTTCTGCTCCCAAGGACTTACAAAGTGAACATTTGTATGTCCCGTATTAA  540
      L S T S A P K G L T K V N I C M S R I K
541 AGTACTTTGAACTCTGTTTCAAGGCTGTTTGGCAATCAAAATGAATGATTTTCACGT  600
      S T L N S V S K A V F G N Q N E M I S R
601 TTAGCTCAATTTAAGCCAGTTCCTCCAAATTTTAAGAAAGATATCGGATAGTGCTGTTA  660
      L A Q F K P S S Q I L R K V S D S G W L
661 AAACAGAAAAACATCAACACAGCCATCAAACTCTGAAAAAATAAGTGACCAATCAGCA  720
      K Q K N I K Q A I K S L K K Y S D K S A
721 GAAAAAGTCTTTTCCAGAGAGAAAAAGTCACTTAAGACAAAGAGAAGATATAGGT  780
      E K S P F P E E K S H I I D K E E D I G
781 AAACGAGTCTTTTTCATTACACAAGTTCTATAACCACAAAAATTTGAGACTCAITTTAC  840
      K R S L F H Y T S S I T T K F G D S F Y
841 TTTTATCAAAATCATATTAATTCAATATTTCAAACGTAAAGGAAAAAATGTCTCAACAAA  900
      F L S N H I N S Y F K R K E K M S Q Q K
901 GAAAAATGAACATTTCCGGGACAAATCAGAACTTGAAGATATAAAAAAGTAGAAGAGG  960
      E N E H F R D K S E L E D K K V E E G K
961 TTAAGATCTCCAGATCCCTGGCAATCCTTGCTTATTAAGCCAGGCTCAGAAATCTGTAC  1020
      L R S P D P G I L A Y K P G S E S V H T
1021 GTGACACAAGCCTACAAGTCTTCTGCGATACCTGATGTTCTTCAAGTTTCAACTAAACA  1080

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FIGURE 1 (SHEET 2)

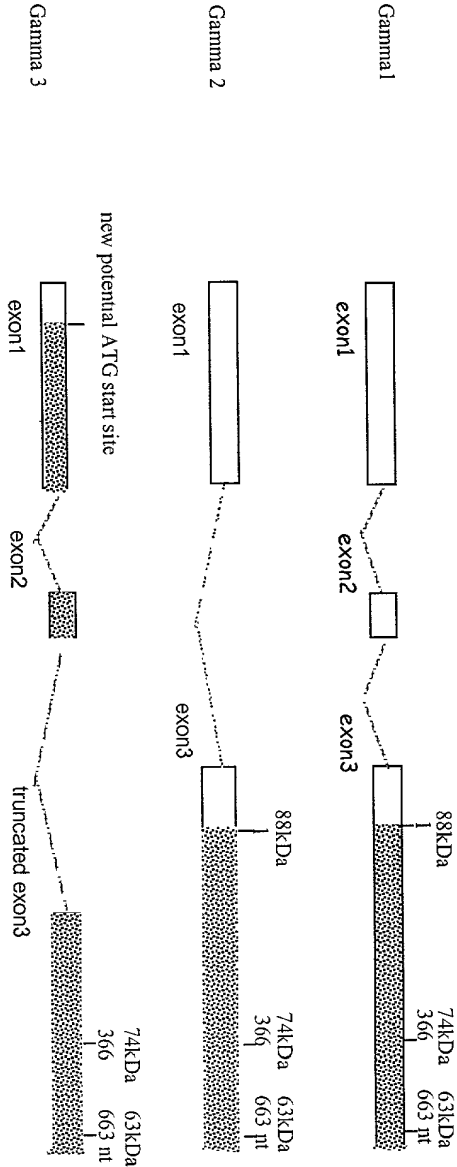
V D K P T S P S A I P D V L Q V S T K Q
1081 AGTATTGCTAACTTTCTTCTCGTCCACGGAAGGTGTACAAGCTTTAGTAGGTAT 1140
S I A N F L S R P T E G V Q A L V G G Y
1141 ATGGTGACTTGTCCTCCCAATTAAAGTATGATTCAAAGTCAGTCAGAAGAACAGGAA 1200
I G G L V P K L K Y D S K S Q S E E Q E
1201 GAGCCTGCTAAAACTGATCAGGCTGTACGCAAGAACAAGAAATGCAGAGAGAAAAGCGT 1260
E P A K T D Q A V S K D R N A E E K K R
1261 TTATCTCTTCAGCGAGAAAAGATTATCGCAAGGGTGAGTATTGATAACAGGACCCGGCA 1320
L S L Q R E K I I A R V S I D N R T R A
1321 TTAGTTCAGGCATTAGAAGAACAACTGACCCAAAGCTCTGCATTACTAGGGTTGAAGAA 1380
L V Q A L R R T T D P K L C I T R V E E
1381 CTGACTTTTCATCTTCTAGAAATTTCTGAAGAAAAGAGTGGCTGTCAAGGAAGAATT 1440
L T F H L L E F P E G K G V A V K E R I
1441 ATTCATATTATTACGACTGAGACAAATTAAAGATGAACCTCTTCAGGCTGCAGTTAGA 1500
I P Y L L R L R Q I K D E T L Q A A V R
1501 GAAATTTTGGCCCTAATTGGCTATGTGATCCAGTGAAAGGAGAGGAATCCGAATTCTC 1560
E I L A L I G Y V D P V K G R G I R I L
1561 TCAATTGATGTGAGGAACAAGGGCGTGTCTCTCCAGACCCTACGAAAATTAGTT 1620
S I D G G G T R G V V A L Q T L R K L V
1621 GAACCTTACTCAGAAGCCAGTTTCATCAGCTCTTTGATTACATTTGTGTGAAGCACAGGT 1680
E L T Q K P V H Q L F D Y I C G V S T G
1681 GCCATATTAGCTTTTCATGTTGGGTTGTTTCATATGCCCTTGATGAATGTAGGAACCTT 1740
A I L A F M L G L F H M P L D E C E E L
1741 TATCGAAAATTAGGATCAGATGTATTTTCACAAAATGTCATTGTTGGAACAGTAAAAATG 1800
Y R K L G S D V F S Q N V I V G T V K M
1801 AGTTGAGCCATGCATTTTATGACAGTCAAAACATGGGAAAACAATTCTTAAGGATAGGATG 1860
S W S H A F Y D S Q T W E N I L K D R M
1861 GGATCTGCACATGATGATTGAACACAGCAAGAAACCCACATGTCTTAAGGTAGCTGTGTA 1920
G S A L M I E T A R N P T C P K V A A V
1921 AGTACCATAGTAATAGAGGATAACACCCAAGCTTTTGTGTTCAAGAACTATGTCAT 1980
S T I V N R G I T P K A F V F R N Y G H
1981 TTTCCTGGAATCAACTCTCATATTTTGGGAGGCTGTCAGTATAAATGTGCAAGGCCATT 2040
F P G I N S H Y L G G C Q Y K M W Q A I

FIGURE 1 (SHEET 3)

2581	TATGCTTATGTTCTCATAAATGAAGGCTCTGTTTGAAGATCAACCCACATTCAATTAAGGA	2640
2641	TTGTGGGGTTTCGACATGAGTTAACTTTGAAATACGTAATCTGGAGAAATCCTGAAAA	2700
2701	AGACGGTGCTTCAACCCAGCTTGCATAGCACAGAGAATATTCTTGGTTACAGAATTCAATAT	2760
2761	GGGAAGTAGGCTTTTAAGATGTTAATAATTAGCTAAAGCTTTAGTAAACCTTACTGTGCTA	2820
2821	GTAGATTTTAGTAGATATTGGTGTATATTGTTGATGTTTGAAAAATATAATTAATATATG	2880
2881	TGCCGAACAAGAAACCGAAAGCTATATTGTACTGTGATTTTACTTTAGTCCCTCATAT	2940
2941	CATGTTGAATTTATGTGATCATTTGATTTTATTTTCATATGGAAAAAGCTAATTTCTCTTAA	3000
3001	ATTTCATTACCTAATATTCTCACTAGCTATGTTCTCCAATCCACACTGCCCTTTTATTTGT	3060
3061	AATATCATCTAAATAGATGCAGAAAAATGGAATTTTCTCTATTTAAAGTATTTTACATTTG	3120
3121	ACATAAAAAAGAACCCAGATACAGTTTTCATTCAGATATGTTTATTTTAAACATTTGTTGG	3180
3180	TTAAAAAAGGTGAAGTTCCAGTCAACCACTTTTAAACCTGAAAAATTTCAAGATTAATGCTA	3240
3241	TATTAACTTTTCCAGATCTAACACTAGCTTATTTCTTCCCTGTTATAAAAATGGTTTGAAC	3300
3301	TACTGAGGAGATATTCCTATCATTTAACAAAAATTAACATAATTAAATAATCTGTTGTTAAA	3360
3361	AGGCTAATGTCATTTTAAAAATTAAATTTTTGTTTCATATAGTAGCTCCCTTTAGCCTTGA	3420

FIGURE 2

iPLA₂ γ Splice Variants



open boxes are noncoding regions
shaded regions are putative coding regions
stippled lines represent intron splicing

FIGURE 3

Splice Variants of iPLA₂ γ

4/5/00

| Exon 1

1 1 TGGAAGCTCAGCTGATGCAGGCCGGTTGGAGTGGACGTCATTGCCGGGAACGAGCGAGTCGCCGCTGCAGCCCTAGTGAC 80
2 2 TGGAAGCTCAGCTGATGCAGGCCGGTTGGAGTGGACGTCATTGCCGGGAACGAGCGAGTCGCCGCTGCAGCCCTAGTGAC
3 3 TGGAAGCTCAGCTGATGCAGGCCGGTTGGAGTGGACGTCATTGCCGGGAACGAGCGAGTCGCCGCTGCAGCCCTAGTGAC
M Q A G W S G R H C R E R A S R R C S P S D

81 | Exon 2

| Exon 3 160

1 1 TCGCGCCTGCATCCCGATTGTCTTCTCCTCCAAGTCTACATGATTACCTGAAGTTTAATAAGTAAGACCATGAATTATG
2 2 TCGCGCCTGCATCCCG.....GTAAGACCATGAATTATG
3 3 TCGCGCCTGCATCCCGATTGTCTTCTCCTCCAAGTCTACATGATTACCTGAAGTTTAATAA.....
C G L H P D C L L L Q G L H D Y L K F N N

161

140

M S I N L

1 GCATTTCTTAATGAAGCGTTCAAGAAGTGAGAGAATGTCATAGAAATTAATGATTTTAAAGTTATGTCATATTAATCTG
2 GCATTTNTTAATGAAGCGTTCAAGAAGTGAGAGAATGTCATANAAATTAATGATTTTAAAGTTATGTCATATTAATCTG
3

141

220

T V D I Y I Y L L S N A R S V C G K Q R S K Q L Y F L
1 ACTGTAGATATATATATTACCTTACCTTAGTAATGCAAGAAGTGTGTTGGGAAGCAGAGAAGCAAGCAACTGTATTCTT
2 ACTGTAGATATATATATTACCTTACCTTAGTAATGCAAGAAGTGTGTTGGGAAGCAGAGAAGCAAGCAACTGTATTCTT
3TAATGCAAGAAGTGTGTTGGGAAGCAGAGAAGCAAGCAACTGTATTCTT
N A R S V C G K Q R S K Q L Y F L

Full-length iPLA₂ γ

Primers for PCR amplification of full-length 88kDa iPLA2 γ :

Sense primer M444 5'-TTTGTGCACATGCTATTAACTGACTGTAGATA-3'

Reverse primer M458	5'-GCATAGCATGCTCACAAATTTGAAAGAATGGAAGTCC-3'
---------------------	---

Sequence of 88kDa iPLA2 gamma:

[illegible]

gtgaagaggggaaatlaagatctccagatccctggcatccctggtcttataagccaggctca
V E E G K L R S P D P G I L A Y K P G S
gaatctgtacataccggtggacaagccctacaagtcctctctgcgataacctgattgtcttcaa
E S V H T V D K P T S P S A I P D V L Q
gtttcaactaaacaaaglatctgaacttcttctctcgtccacgggaaggtgtacaagct
V S T K Q S I A N F L S R P T E G V Q A
ttaagtagtggttatatattggtgactgttccccaatlaagtatgatccaagagtcag
L V G G Y I G G L V P K L K Y D S K S Q
tcagaagaacagggaagagcctgtctaaactgatcaggctgtcagcaagacagaatgca
S E E Q E E P A K T D Q A V S K D R N A
gaagagaaaaagcgtttatctctcagcgcgagaaaagattatcgcaaggygtgagtatgat
E E K K R L S L Q R E K I I A R V S I D
aacaggagccccgggcattagttcaggccattaaagaacaaactgacccaagctctgcatt
N R T R A L V Q A L R R T T D P K L C I
actagggttgaagaactgactgttttcattctctagaattctctgaaggaagaaggagtgct
T R V E E L T F H L L E F P E G K G V A
gtcaagguaagaattatccatatttattacgactgagacaaatlaaggatgaactctt
V K E R I I P Y L L R L R Q I K D E T L
caggctgcagttagagaaatttggccctaattggtctatgttgatccagtgaaagggaga
Q A A V R E I L A L I G Y V D P V K G R
ggatccgaattctctcaattgatggtggaggaacaaggygcgtggtctctccagacc
G I R I L S I D G G T R G V V A L Q T
ctacgaaaaattagttgaacttactcagaagccagttcatcagctcttgattacattgt
L R K L V E L T Q K P V H Q L F D Y I C
ggtgtgaagcacaggtgcatattagcttccattggtgggttttcataatgcccctggat
G V S T G A I L A F M L G L F H M P L D
gaatgtgagaacttatacgaaaattaggatcagatgtattttcacaaaatgtcattgt
E C E E L Y R K L G S D V F S Q N V I V
ggaaccaqtaaaaatgagttggagccatgcatlttlatgacaggtcaaacatggygaaaacat
G T V K M S W S H A F Y D S Q T W E N I
cttaaggatagatgggatctgcacactgatgatggaacagcaagaaacccacatgtcct
L K D R M G S A L M I E T A R N P T C P
aaggtagctgtctgaagttaccatagtaatatagagggataaacacccaagaagcttctgtctc

FIGURE 4 (SHEET 3)

K V A A V S T I V N R G I T P K A F V F
agaactatggtcatttctcgtgaatcaactcattatttggagcgtgcagtataaa
R N Y G H F P G I N S H Y L G G C Q Y K
atgtggcaggccattagagccctcatctgtgtccaggctactttgcagaatatgcatg
M W Q A I R A S S A A P G Y F A E Y A L
ggaatatgatccttcatcaagatggaggtttgccttctgaataacccttcggcattagctatg
G N D L H Q D D G G L L L N N P S A L A M
catgagtgtaaatgtcttggccagatgtgcccgttagagtgcatagtatccctgggcact
H E C K C L W P D V P L E C I V S L G T
ggacgttatgagagtgatgtgagaaacacggtaacatcacacaagcttgaaactaaactt
G R Y E S D V R N T V T Y T S L K T K L
tctaattgttatccaacagtgctacagatatcagaagaagtcacatatatgttgatgacctg
S N V I N S A T D T E E V H I M L D G L
ttacctcctgacacctatttttagattccaatccctgttaattgttgaaacacatcaccttagat
L P P D T Y F R F N P V M C E N I P L D
gaaagtcgaaatgaaaagctggatcagctgcagtttggaagggttgaaatacatagaaga
E S R N E K L D Q L Q L E G L K Y I E R
aatgaacaaaaaatgaaaaaagltgcaaaaatattaagtcagaagaaaaaaccaactctgcag
N E Q K M K K V A K I L S Q E K T T L Q
aaaattaatgattggataaaaattaaaactgatatgtatgaaggacttccattctttcca
K I N D W I K L K T D M Y E G L P F F S
aaattgtga
K L -

FIGURE 5 (SHEET 1)

Sequence of 77kDa iPLA2 gamma:
starting at amino acid 101 (nucleotide 301)

Primers for PCR amplification of 77kDa iPLA2γ:

Sense primer m534	5'-TGAACGTCGACATGTCGCCGTATTAAAA-3'
Reverse primer M458	5'-GCATAGCATGCTCACAATTGTGAAAAGAATGGAAGTCC-3'

atgtcccgatttaaaagtaacttgaactctgtttccaaggctgttttggcaatcaaat
M S R I K S T L N S V S K A V F G N Q N
gaaatgatttcacgtttagctcaatttaagccaagttccccaattttaagaaagltacg
E M I S R L A Q F K P S S Q I L R K V S
gatagtgctgtttaaaaacagaaaaacatcaaaacagccatcaaatctctgaaaaaatat
D S G W L K Q K N I K Q A I K S L K K Y
agtgcacaatcagcagaaaagagtcctttccagaagagaaaagtcacattatagacaaa
S D K S A E K S P F P E E K S H I I D K
gaagaagatatagtaaacgcgactcttttcatlacacaagttctataaccacaaaattt
E E D I G K R S L F H Y T S S I T T K F
ggagactcattctactttttatccaatcatcatattcatcatatttcaaacgtaaggaaaaa
G D S F Y F L S N H I N S Y F K R K E K
atgtctcaacaaaaggaatatgaacatttccgggacaatcagaacttgaagataaaaag
M S Q Q K E N E H F R D K S E L E D K K
gtagaagagggaattaagatctccagatcctggcatcctgtgtataagccaggtca
V E E G K L R S P D P G I L A Y K P G S
gaatctgtacatacgtggaacaagcctacaagtccttctgcatacctgatgttcttcaa
E S V H T V D K P T S P S A I P D V L Q
gtttcaactaaacaagtattgtctaacttttctctcgtccacggaaggtgtacaagct
V S T K Q S I A N F L S R P T E G V Q A
ttagtagtggttatattgtgtgacttgtccccaatttaagtatgatccaagagtcag
L V G G Y I G G L V P K L K Y D S K S Q
tcagaagaacaggaagagcctgtctaaaactgatcaggtgttcagcaaaagacagaatgca
S E E Q E E P A K T D Q A V S K D R N A

FIGURE 5 (SHEET 2)

G R Y E S D V R N T V T Y T S L K T K L
tctaattgttatcaacagctgtctacagatatcagaagaagtcataatgtctgtatgycctg
S N V I N S A T D T E E V H I M L D G L
ttacctccctgacaccctattttagattcaatccctgtaatgtgtgaacacatacccttagat
L P P D T Y F R F N P V M C E N I P L D
gaaagtcgaaatgaaaagctggtacagctgcagttggaaaggttgaatatcatagaaga
E S R N E K L D Q L Q L E G L K Y I E R
aatgaacacaaaatgaaaaaagttgcacaaaatattaagtcaagaaaaaaccaactctgcag
N E Q K M K K V A K I L S Q E K T T L Q
aaaattaatgattggtataaatlaaaaactgatatgtatgaaggaacttcattctttca
K I N D W I K L K T D M Y E G L P F F S
aaattgtga
K L -

FIGURE 6 (SHEET 1)

Sequence of 74kDa iPLA2 gamma:
starting at amino acid 122 (nucleotide 364)

Primers for PCR amplification of 74kDa iPLA2:

Sense primer m533	5'- TCAAGTCGACATGATTTCACGTTAGC -3'
Reverse primer M458	5'-GCATAGCATGCTCACCAATTTGAAAGAATGGAAGTCC-3'

atgatttcacgcttagctcaatttaagccaagttcccaattttaagaaaagtcg
M I S R L A Q F K P S S Q I L R K V S
gatagtgctggttaaaacagaaaaacatccaacaagccatcaatctctgaaaaatat
D S G W L K Q K N I K Q A I K S L K K Y
agtgaacaatcagcagaaaaagagtcctttccagaagagaaaaagtcacattatagacaaa
S D K S A E K S P F P E E K S H I I D K
gaagaaagatatagtaaacgcagtcctttttcattacacaagttctataaccacaaaattt
E E D I G K R S L F H Y T S S I T T K F
ggagactcattctactttttatccaatcataatcaattcaatttcaaacgtaagyaaaaa
G D S F Y F L S N H I N S Y F K R K E K
atgtctcaacaaaaggaatatgaacatttccggygacaatcagaacttgaagataaaaag
M S Q Q K E N E H F R D K S E L E D K K
gtagaagagggaaattaaagatctccagatcctgcatcctgcttataagccaggtcca
V E E G K L R S P D P G I L A Y K P G S
gaatctgtacatagcgttgacaagcctacaagtccttctgcatacctgatgttcttcaa
E S V H T V D K P T S P S A I P D V L Q
gtttcaactaaacaagtattgtctaacttcttctcgtcccaaggaagtgtaacaagct
V S T K Q S I A N F L S R P T E G V Q A
ttagtagtggttatattggttgacttgcccccaattaaagtatgttcaaaagagtcag
L V G G Y I G G L V P K L K Y D S K S Q
tcagaagaacaggaagagcctgtctaaactgatccagctgtcagcaagaagacagaatgca
S E E Q E E P A K T D Q A V S K D R N A
gaggagaaaaagcgtttatctcttcagcgagaaaaagattatcgcaaggtgagtatgat
E E K K R L S L Q R E K I I A R V S I D

FIGURE 6 (SHEET 2)

aacaggaccgggcatagttcaggcattagaagaacaactgacccaagctctgcatt
N R T R A L V Q A L R R T T D P K L C I
actaggtttgaagaactgacttttcattctcttagaatttcctgaaggaaaggtgct
T R V E E L T F H L L E F P E G K G V A
gtcaaggaaagaattatccatatttattaccgactgagacaattagatgaactct
V K E R I I P Y L L R L R Q I K D E T L
caggctgcagttagagaatlttggccctaattggtatgtgattccagtgaaaggaga
Q A A V R E I L A L I G Y V D P V K G R
ggaatccgaattctctcaattgatgtgtgaggaacaagggcgtgtgtctctccagacc
G I R I L S I D G G T R G V V A L Q T
ctacgaaaattagttgaacttactcagaagccagttcatcagctcttgattacatltgt
L R K L V E L T Q K P V H Q L F D Y I C
ggtgtgaagcacaggtgccaattagcttctcatgtttgggtgtttcatatgcccctgat
G V S T G A I L A F M L G L F H M P L D
gaatgtgagaaactttatcggaaaattagatcagatgtattttcacaaaatgtcatgtt
E C E E L Y R K L G S D V F S Q N V I V
ggaacagtaaaaatgagttggagccatgcattttatgacagtcacaacatgygaaaacatt
G T V K M S W S H A F Y D S Q T W E N I
cttaaggatagatggatctgcactgatgatltgaacacgacaagaaacccacatgtcct
L K D R M G S A L M I E T A R N P T C P
aaggtagctgtgtaagtagccatagtaaatagaggataacacccaagcttltgtgtc
K V A A V S T I V N R G I T P K A F V F
agaaactatgtcatlttctctggaatcaactctcatattttgggaggtgtcagltataaa
R N Y G H F P G I N S H Y L G G C Q Y K
atgtggcagggccattagagcctcatctgtctcgcaggtcactttgcaggaatatgcattg
M W Q A I R A S S A A P G Y F A E Y A L
ggaatatgatcttcacagaatggaggttgcctctgaataaccctcggcattagctatg
G N D L H Q D G G L L L N N P S A L A M
catgagtgtaaatgtcttggccagatgtgcccgttagagtgcataglatccctgggcact
H E C K C L W P D V P L E C I V S L G T
ggacgttatgagatgtatgtgagaacaacggttaacatacacaagcttgaaaactaacct
G R Y E S D V R N T V T Y T S L K T K L
tctaattgtatacaacagtgctacagatatcagaagaagtcacataatatgtcttgatgycctg

FIGURE 6 (SHEET 3)

S N V I N S A T D T E E V H I M L D G L
ttacctcctgacacctattttagatccaatccctgtaatgtgaaacataaccttagat
L P P D T Y F R F N P V M C E N I P L D
gaaagtcgaaatgaaaagctggatcagctgcagttggaaggttgaatatacatagaaga
E S R N E K L D Q L Q L E G L K Y I E R
aatgaacaaaaaatgaaaaaagtgcacaaatatgaagtcagaaaaaaccaacttgcag
N E Q K M K K V A K I L S Q E K T T L Q
aaaatgaatgattggataaaaatgaactgatatgtatgaaggacttccatttcca
K I N D W I K L K T D M Y E G L P F F S
aaattgtga
K L -

FIGURE 7 (SHEET 1)

Sequence of 63kDa iPLA2 gamma

starting at amino acid 221 (nucleotide 661)

Sense primer M530

5'-GTAGTCGACAATGTTCTCACCAAAGG-3'

Reverse primer M458

5'GCATAGCATGCTCACAAATTTGAAAGAATGGAAGTCC-3')

atgtctcaacaaaggaataatgaacatttccgggacaaatcagaacttgaagataaaaaag
M S Q Q K E N E H F R D K S E L E D K K
gtagaagagggaattatagatctccagatcttggcatcctgtgtataaagccaggtca
V E E G K L R S P D P G I L A Y K P G S
gaatctgtacatacgttggacaagcctacaagtccttctctgcgatacctgatgttctcaa
E S V H T V D K P T S P S A I P D V L Q
gtttcaactaaacaaagtattgtctaacttcttctctctgtccacggaagtygtacaagct
V S T K Q S I A N F L S R P T E G V Q A
ttagtagtgtgtattatattgtgtgacttgtccccaaatlaagtatgtatccaagagtcag
L V G G Y I G G L V P K L K Y D S K S Q
tcagaagaacaggaagagcctgtctaaactgtatcaggtgttcagcaagacagaatatgca
S E E Q E E P A K T D Q A V S K D R N A
gaggagaaaaagcgtttatctcttcacgcgagaaaaagattatcgcgaaggttgatgtgat
E E K K R L S L Q R E K I I A R V S I D
aacaggagccccggcattagttcagycattaaagaagaacaactgacccaaaagctctgcatt
N R T R A L V Q A L R R T T D P K L C I
actaggtgtgaagaactgacttcttcattctctctaagaattcctgaagaaaaaggagtgcct
T R V E E L T F H L L E F P E G K G V A
gtcaaggaagaatattatccatatatttaccagctgagacaatlaagvatgaaactctt
V K E R I I P Y L L R L R Q I K D E T L
caggtctgcagtttagagaatatttggccctcaattggtctatgttgatccagtgaaaggagaa
Q A A V R E I L A L I G Y V D P V K G R
ggaatccgaattctctcatttgatgtgtgaggaacaagggcgtygtgtctctccagacc
G I R I L S I D G G T R G V V A L Q T
ctacgaaaattagttgaacttactcagaagaccagttcatccagctcttggattacatttgt
L R K L V E L T Q K P V H Q L F D Y I C

ggtgttaagccacgagtgcacatatagcttccatgltgtgggtgtttccatatgccccttgat
 G V S T G A I L A F M L G L F H M P L D
 gaatgtgaggaaccttattcggaaaaattagatcagatgttatttccacaaaatgltcatgtt
 E C E E L Y R K L G S D V F S Q N V I V
 ggaacagtaaaaaatgagttggagcccatgcatlittatgacagtcacaacatgygaaaacat
 G T V K M S W S H A F Y D S Q T W E N I
 ctaagagatagatggtggtatctgcactgatatgtgaacacgacaagaaaccccatgtcct
 L K D R M G S A L M I E T A R N P T C P
 aagtagctgtctgtaagtaaccatagtaaatagaggutaacaccccaagccttgtgttc
 K V A A V S T I V N R G I T P K A F V F
 agaaacctatgtcatcttccctggaatcaactcctcatatttgggagctgtcagltataaa
 R N Y G H F P G I N S H Y L G G C Q Y K
 atgtggcagggccattagagccctcatctgtctccaggtcactttgcagaatatgcatg
 M W Q A I R A S S A A P G Y F A E Y A L
 ggaatgatcttcatacgaatgtaggttctgtctgaataacccttcggcattagctatg
 G N D L H Q D D G G L L L N N P S A L A M
 catgagtgttaaatgltccttggccagatgtgcccgttagagtgcatagtatccctgggcact
 H E C K C L W P D V P L E C I V S L G T
 ggcacttattgagagtgatgttgagaacacgytlaacatcacacaagcttgaacactaaact
 G R Y E S D V R N T V T Y T S L K T K L
 tctaattgttatacaacagtgctacagatatcagaagaagtcacatatatgtctgtagtgcctg
 S N V I N S A T D T E E V H I M L D G L
 ttacctcctgacacccattttagattcaatccctgtgaatgttgaaaaacatacctctagat
 L P P D T Y F R F N P V M C E N I P L D
 gaaagtcgaaatgnaaaagctggtacagctgcagcttggaaaggttgaatatacatagaaga
 E S R N E K L D Q L Q L E G L K Y I E R
 aatgaacaaaaaatgnaaaaaagttgcnaaaatatlaagtcaagaaaaaacaactctgcag
 N E Q K M K K V A K I L S Q E K T T L Q
 aaaaattaatgattggtataaaattaaaaaactgatatgtatgaaggacttccatltcttca
 K I N D W I K L K T D M Y E G L P F F S
 aaattgtga
 K L -

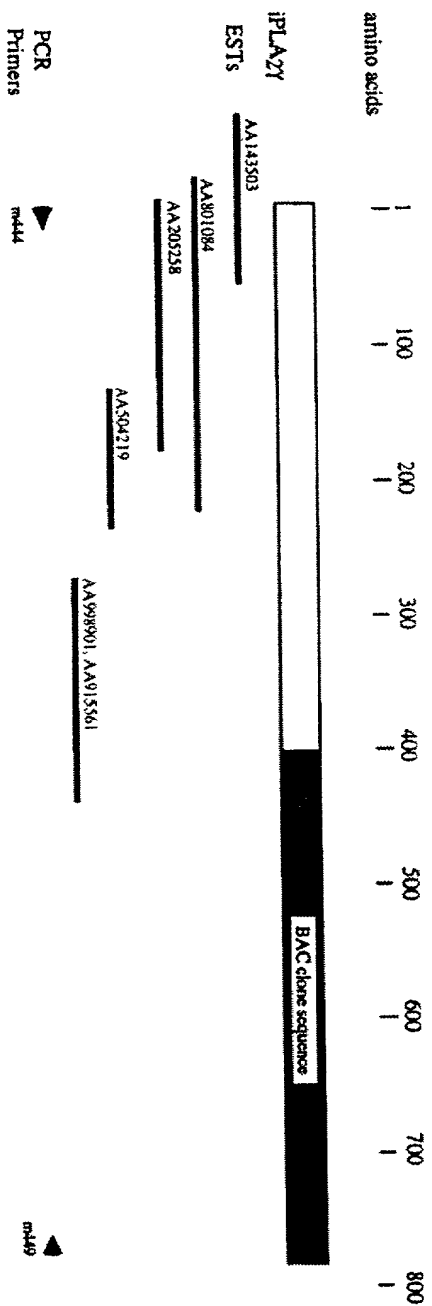


Fig. 8

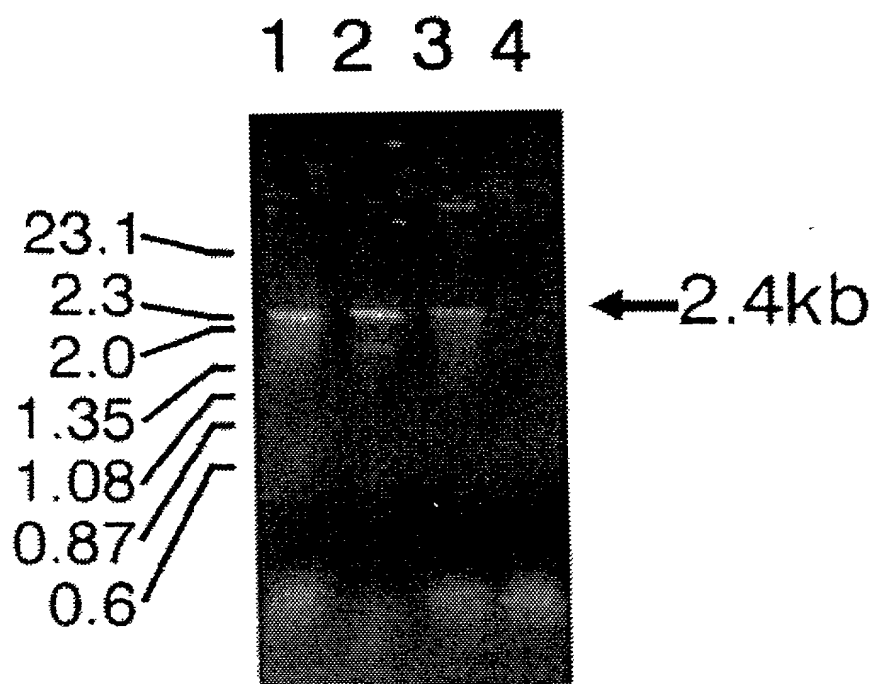
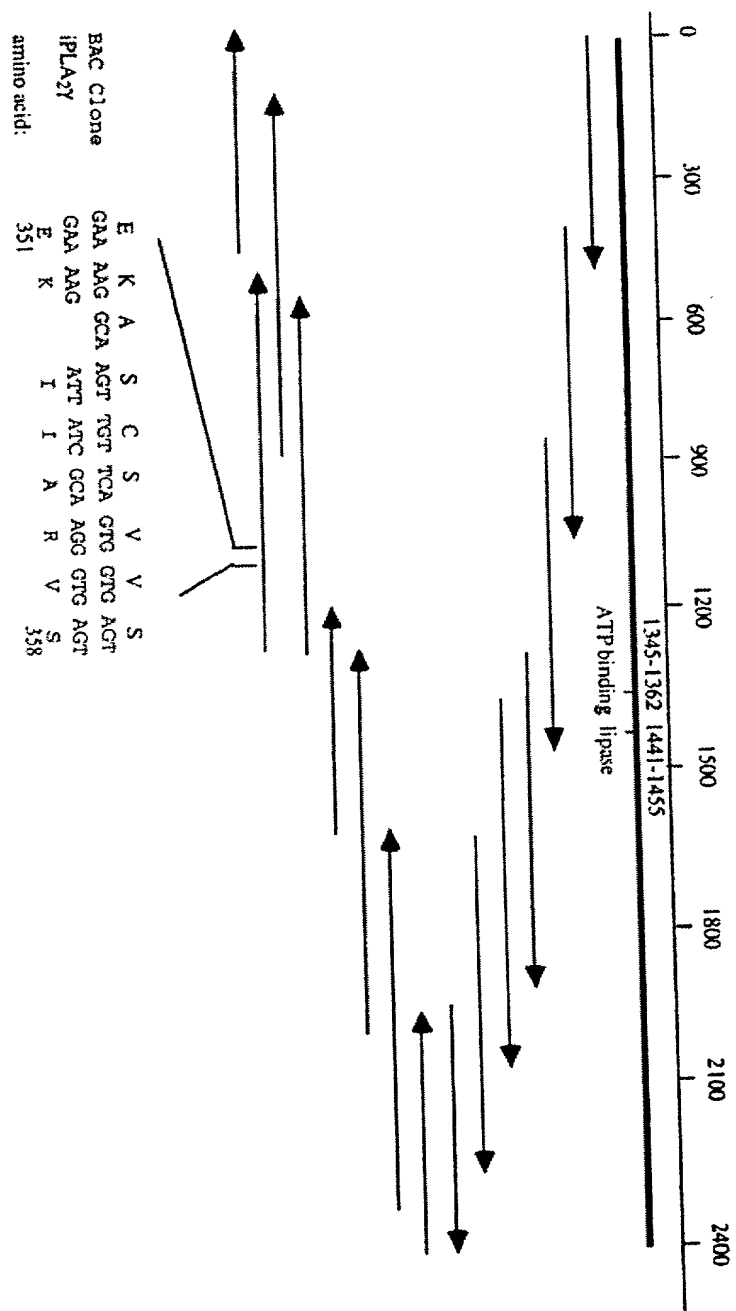


FIG. 9



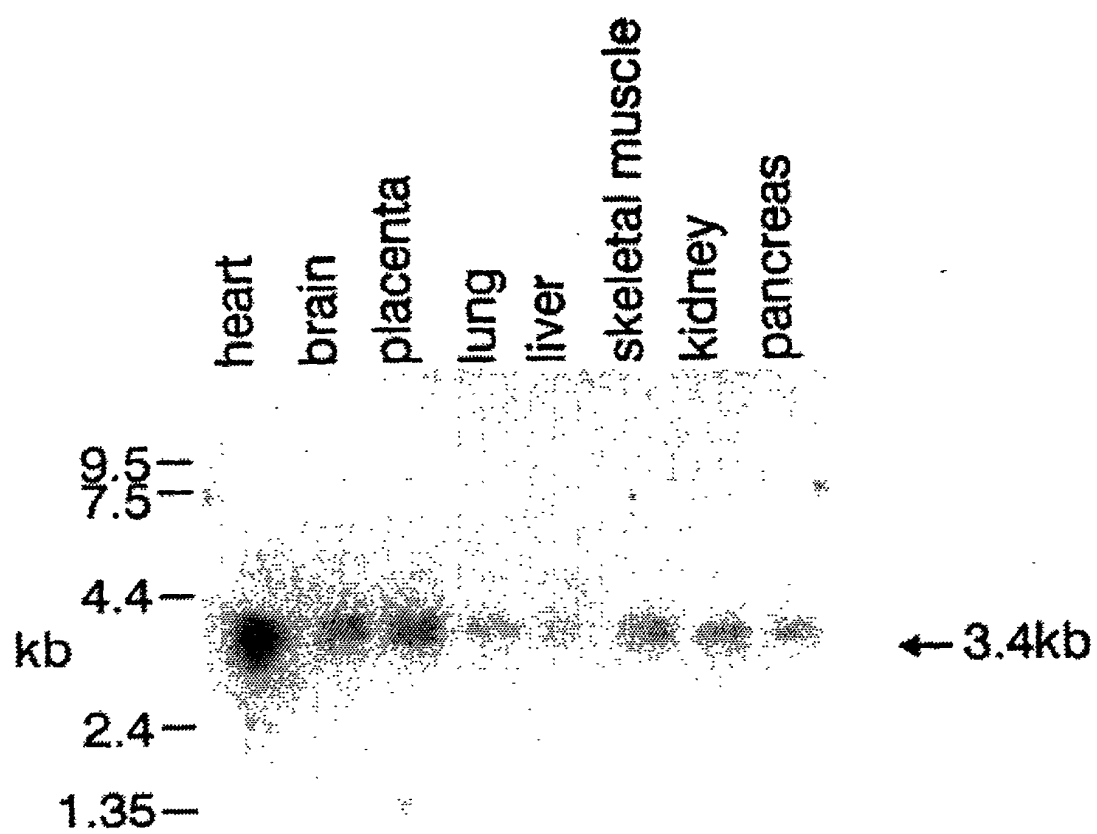


FIG. 12

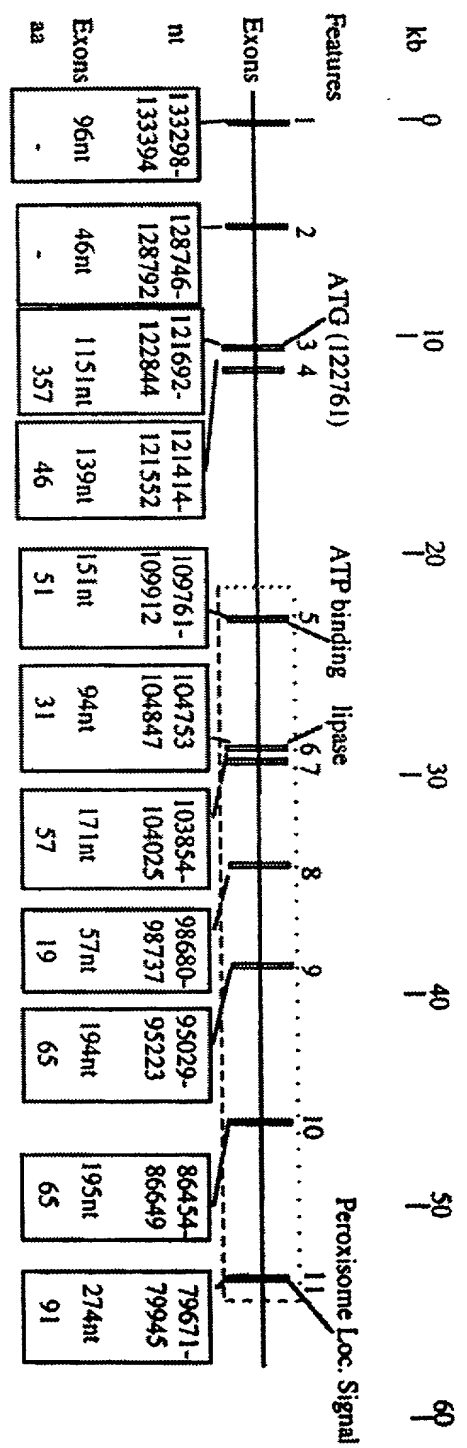


FIG. 13

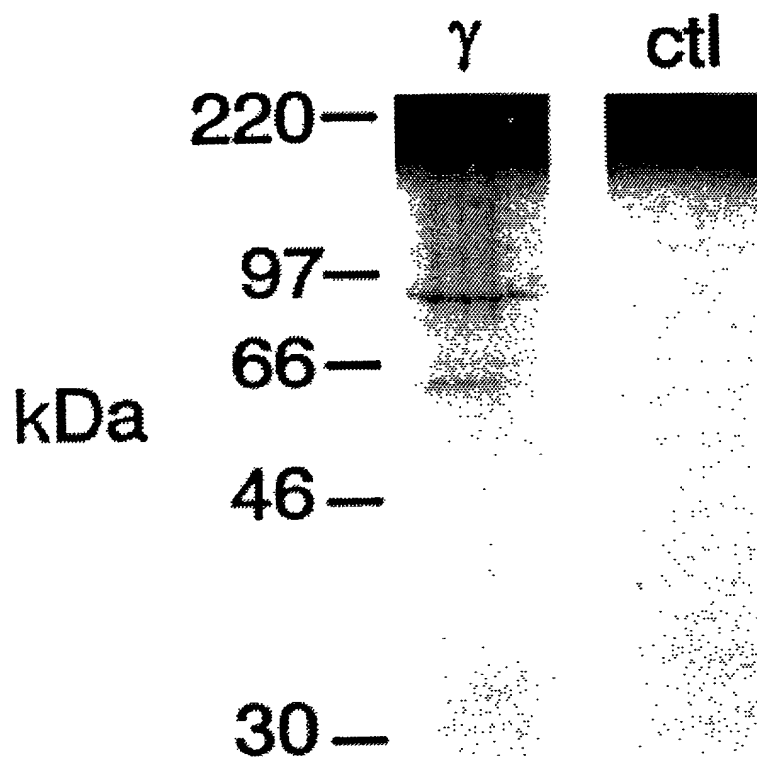


FIG. 14

Parameter	Value	Unit	Source
α	0.0000	deg	Galactic
δ	0.0000	deg	Galactic
β	0.0000	deg	Galactic
γ	0.0000	deg	Galactic
η	0.0000	deg	Galactic
θ	0.0000	deg	Galactic
ϕ	0.0000	deg	Galactic
ψ	0.0000	deg	Galactic
χ	0.0000	deg	Galactic
ω	0.0000	deg	Galactic
ν	0.0000	deg	Galactic
μ	0.0000	deg	Galactic
λ	0.0000	deg	Galactic
κ	0.0000	deg	Galactic
ι	0.0000	deg	Galactic
\hbar	0.0000	deg	Galactic
g	0.0000	deg	Galactic
f	0.0000	deg	Galactic
e	0.0000	deg	Galactic
d	0.0000	deg	Galactic
c	0.0000	deg	Galactic
b	0.0000	deg	Galactic
a	0.0000	deg	Galactic
z	0.0000	deg	Galactic
y	0.0000	deg	Galactic
x	0.0000	deg	Galactic
w	0.0000	deg	Galactic
v	0.0000	deg	Galactic
u	0.0000	deg	Galactic
t	0.0000	deg	Galactic
s	0.0000	deg	Galactic
r	0.0000	deg	Galactic
q	0.0000	deg	Galactic
p	0.0000	deg	Galactic
o	0.0000	deg	Galactic
n	0.0000	deg	Galactic
m	0.0000	deg	Galactic
l	0.0000	deg	Galactic
k	0.0000	deg	Galactic
j	0.0000	deg	Galactic
i	0.0000	deg	Galactic
h	0.0000	deg	Galactic
g	0.0000	deg	Galactic
f	0.0000	deg	Galactic
e	0.0000	deg	Galactic
d	0.0000	deg	Galactic
c	0.0000	deg	Galactic
b	0.0000	deg	Galactic
a	0.0000	deg	Galactic
z	0.0000	deg	Galactic
y	0.0000	deg	Galactic
x	0.0000	deg	Galactic
w	0.0000	deg	Galactic
v	0.0000	deg	Galactic
u	0.0000	deg	Galactic
t	0.0000	deg	Galactic
s	0.0000	deg	Galactic
r	0.0000	deg	Galactic
q	0.0000	deg	Galactic
p	0.0000	deg	Galactic
o	0.0000	deg	Galactic
n	0.0000	deg	Galactic
m	0.0000	deg	Galactic
l	0.0000	deg	Galactic
k	0.0000	deg	Galactic
j	0.0000	deg	Galactic
i	0.0000	deg	Galactic
h	0.0000	deg	Galactic
g	0.0000	deg	Galactic
f	0.0000	deg	Galactic
e	0.0000	deg	Galactic
d	0.0000	deg	Galactic
c	0.0000	deg	Galactic
b	0.0000	deg	Galactic
a	0.0000	deg	Galactic
z	0.0000	deg	Galactic
y	0.0000	deg	Galactic
x	0.0000	deg	Galactic
w	0.0000	deg	Galactic
v	0.0000	deg	Galactic
u	0.0000	deg	Galactic
t	0.0000	deg	Galactic
s	0.0000	deg	Galactic
r	0.0000	deg	Galactic
q	0.0000	deg	Galactic
p	0.0000	deg	Galactic
o	0.0000	deg	Galactic
n	0.0000	deg	Galactic
m	0.0000	deg	Galactic
l	0.0000	deg	Galactic
k	0.0000	deg	Galactic

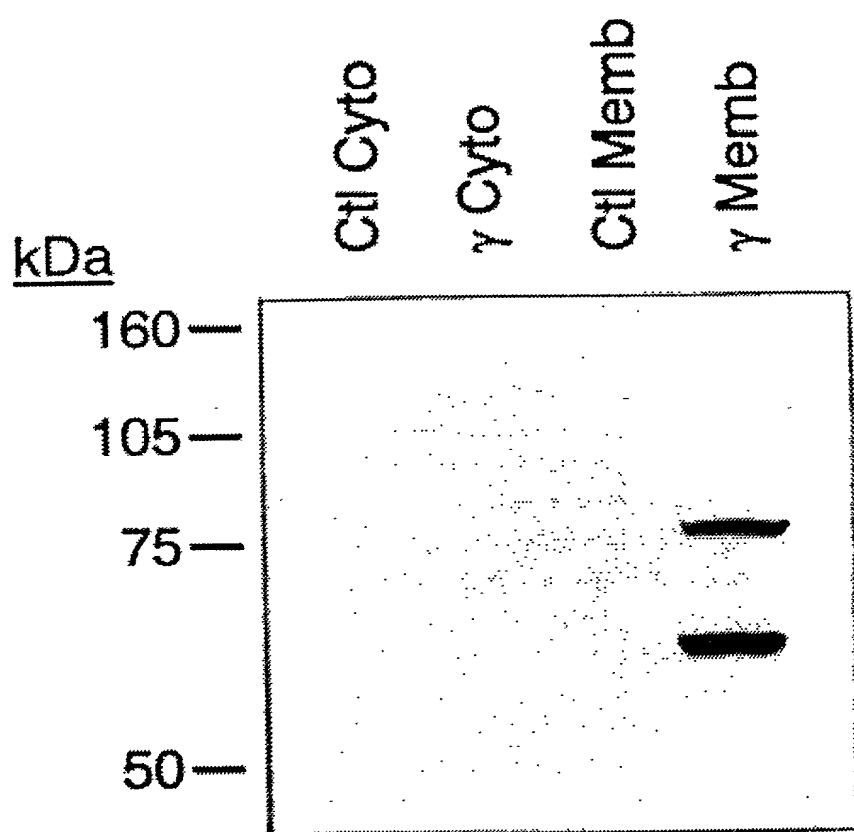


FIG. 15

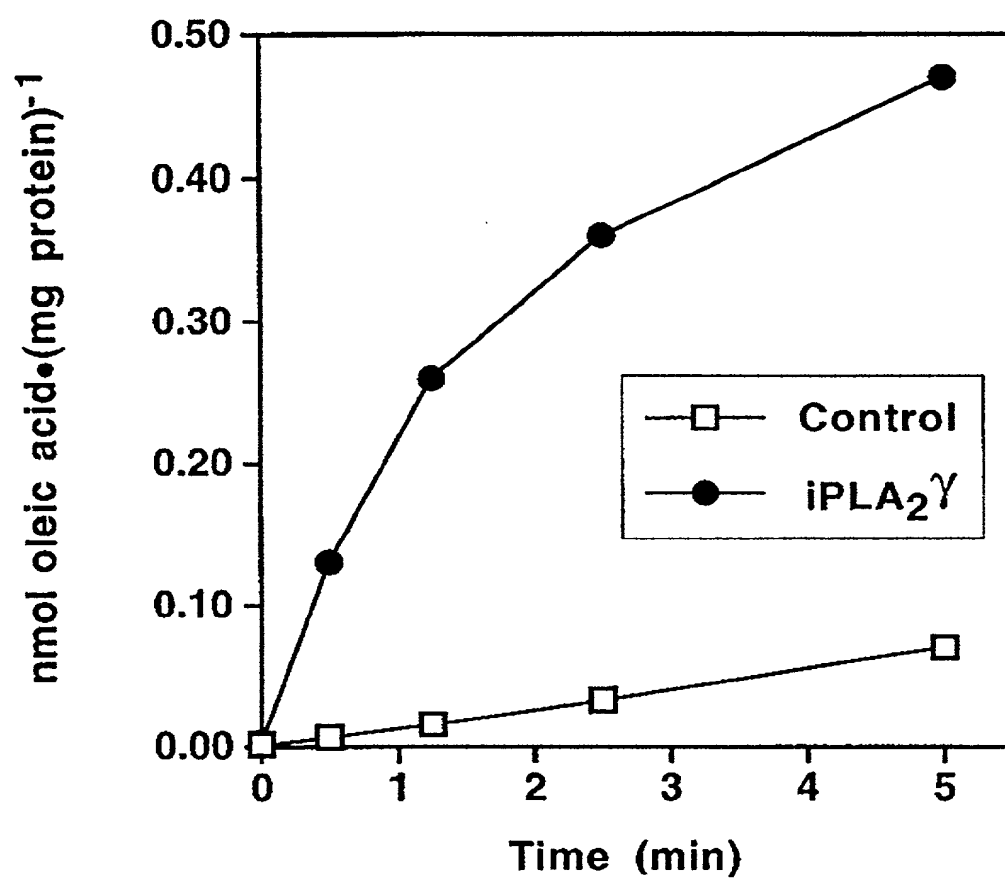


FIG. 16

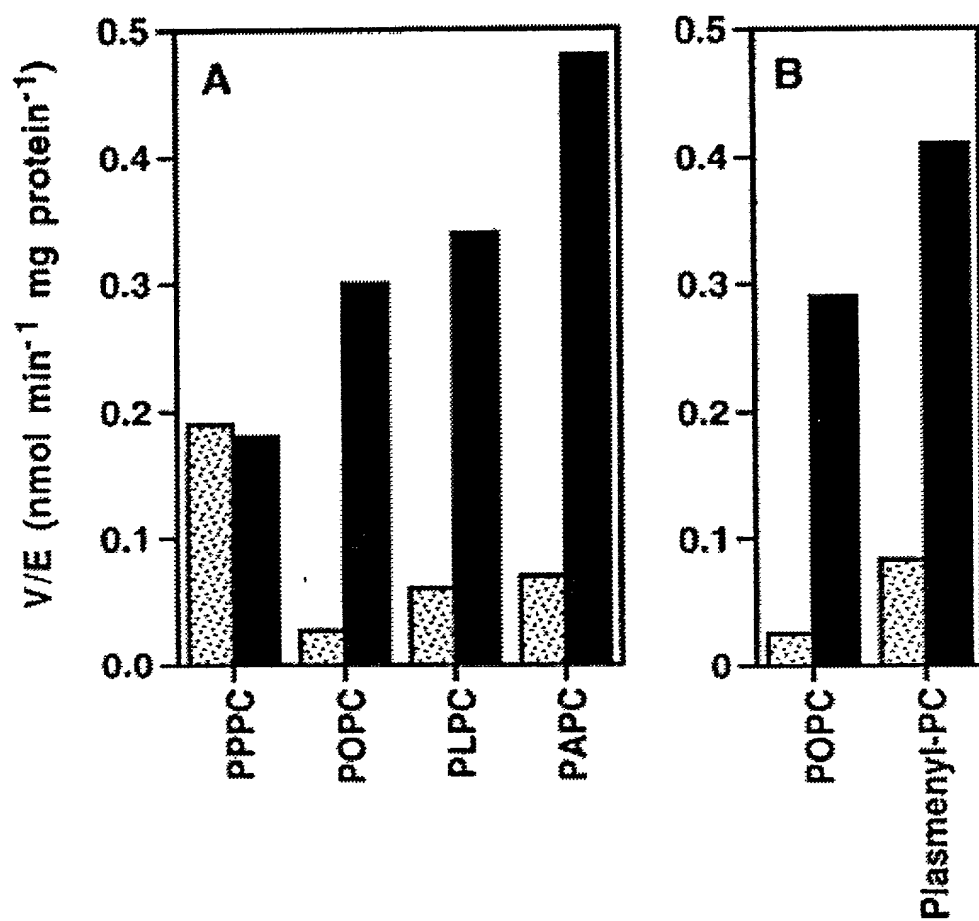


FIG. 17

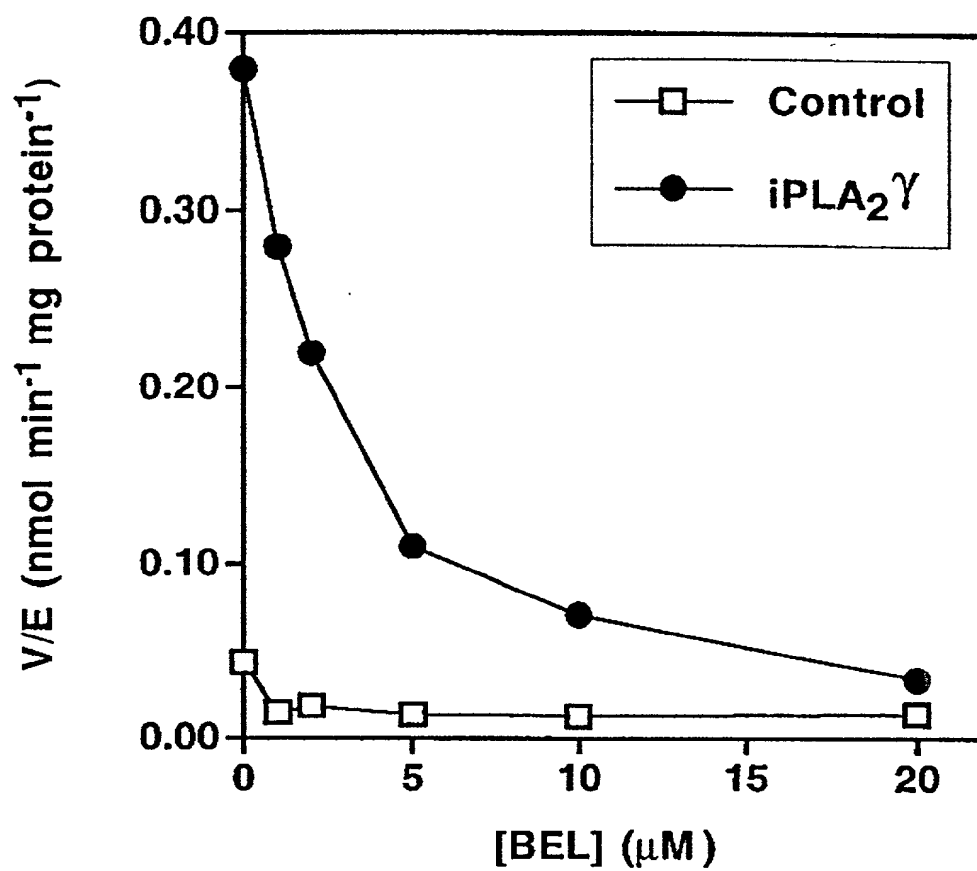


FIG. 18

Translational Repression of iPLA₂ γ

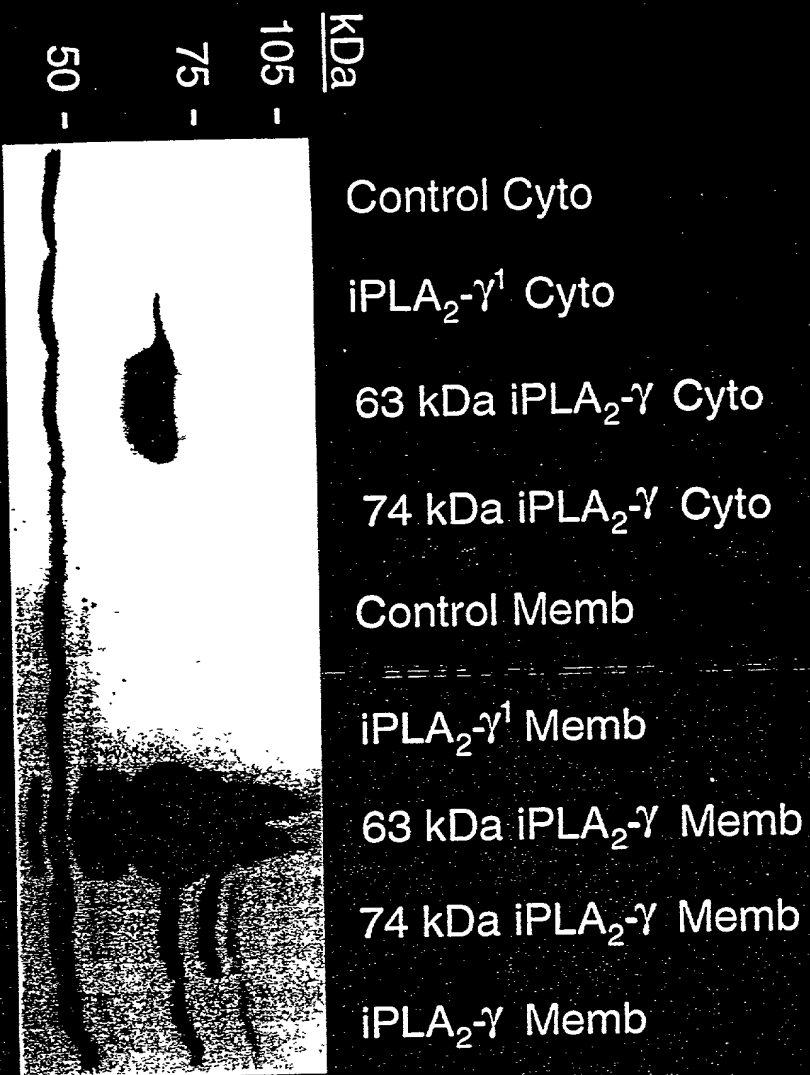


FIG. 19

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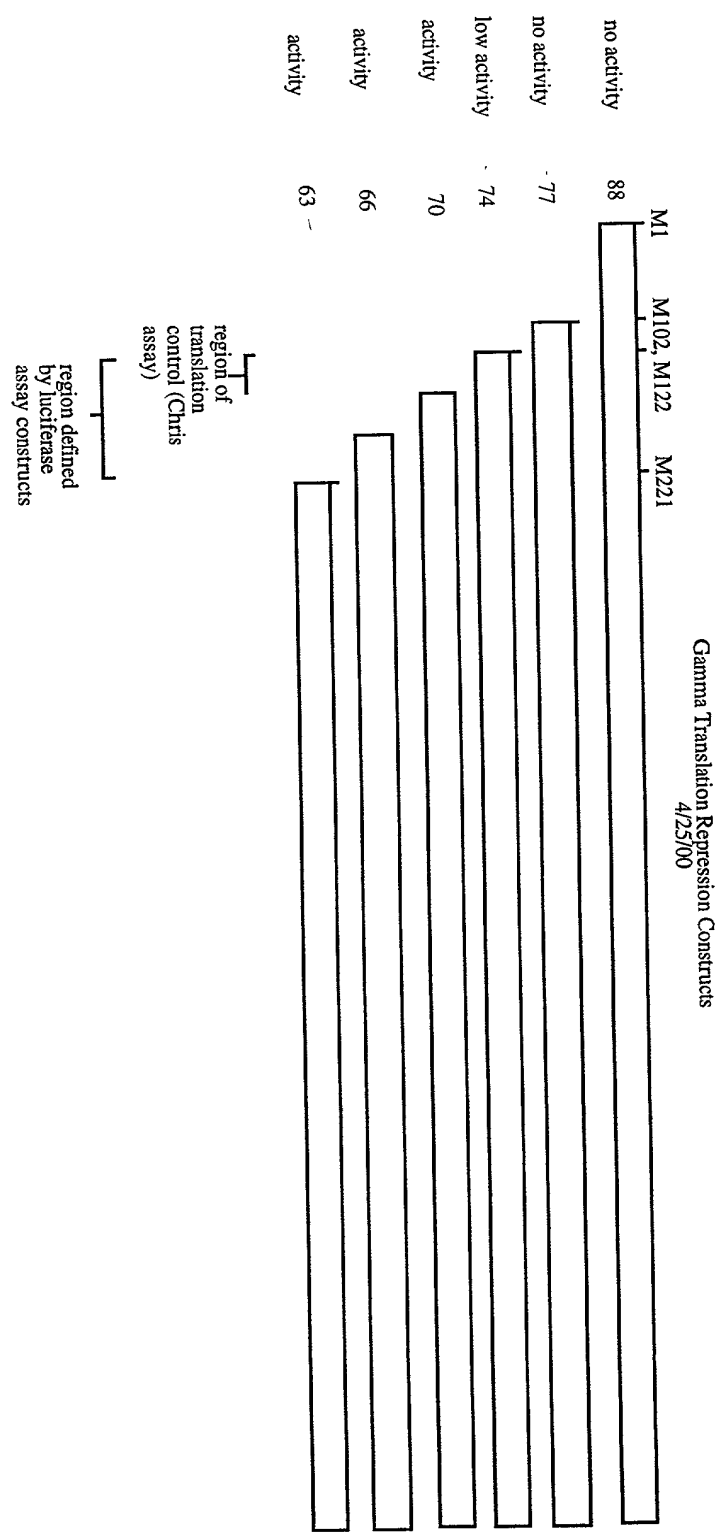


FIG. 20

FIG. 20 is a horizontal bar chart showing the results of a gamma translation repression assay for various constructs. The y-axis lists the constructs: M1, M102, M122, and M221. The x-axis represents the percentage of protein expression, with values 88, 77, 74, 70, 66, and 63. The constructs are grouped into two categories: 'no activity' (M1, M102) and 'low activity' (M122, M221). A bracket on the right indicates the 'region of translation control (Chris assay)' for constructs M102, M122, and M221. Another bracket on the right indicates the 'region defined by luciferase assay constructs' for constructs M102, M122, and M221.

Additionally, iPLA2 γ sequences were inserted by ligation of 15-23mer annealed phosphorylated oligonucleotide pairs 5' of full-length luciferase coding sequence cloned into pFASTBAC via NotI/XbaI restriction sites and then luciferase activity of recombinant protein produced in the Sf9 system was subsequently measured using the Luciferase Assay System of Promega.

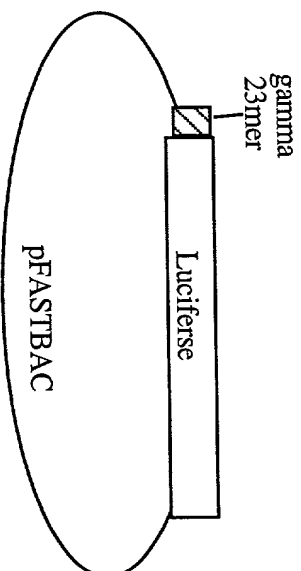


FIG. 21

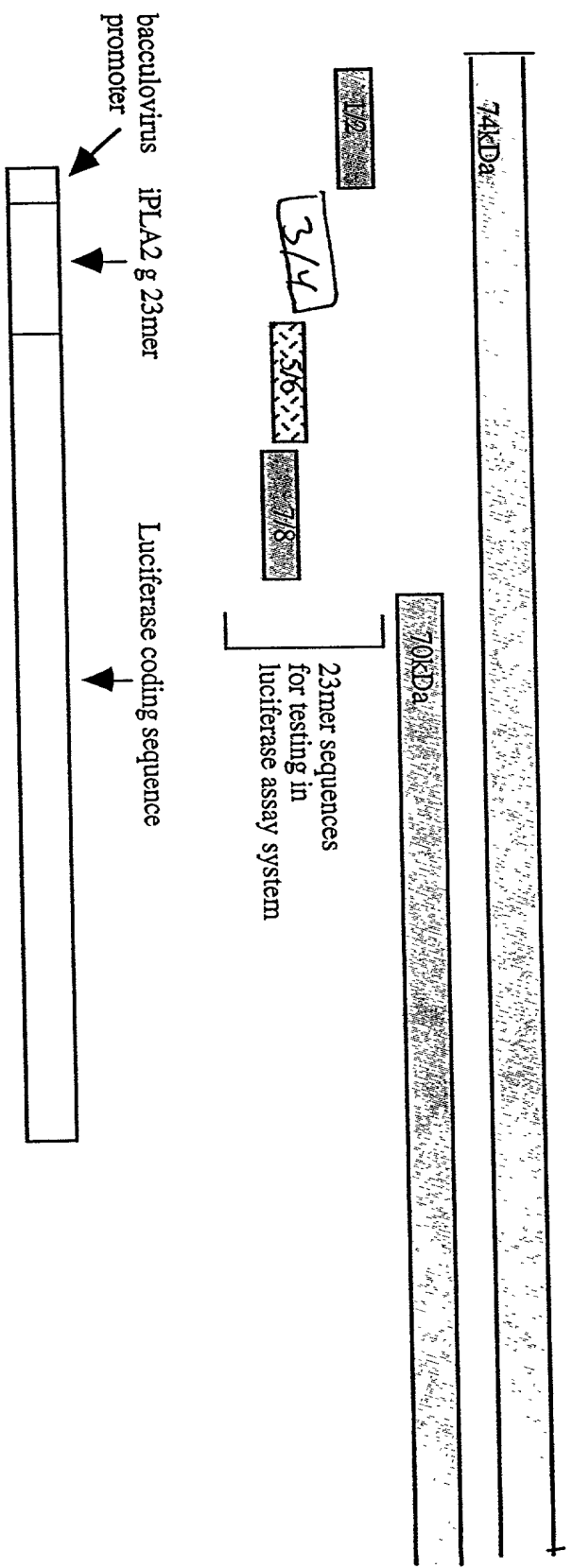


FIG. 22

**phosphorylated oligo pairs for sequence between nucleotide 364- 455
for translational repression
of iPLA2 gamma in the luciferase expression system:**

iPLA2γ	atgattccacggttagctcaatttaagccaagttccccaattttaagaaaagtatcggatagtggtggttaaaaacagaaaacatcaaaaca
1/2	tcgacctgatttcacggttagctcaatt ggactaaagtgcaaatcgaggttaaccgg
3/4	tcgactaagccaagttcccaattttaa gattcgggttcaagggtttaaaattccgg
5/6	tcgacgaaaagtatcggatagtggtg gctttcatagcctatcacgaccccg
7/8	tcgacttaaaacagaaaaacatcaaca gaatttgtctttttagtttgtccgg

FIG. 23

FIG. 23 shows the sequence of the phosphorylated oligo pairs for sequence between nucleotide 364- 455 for translational repression of iPLA2 gamma in the luciferase expression system.

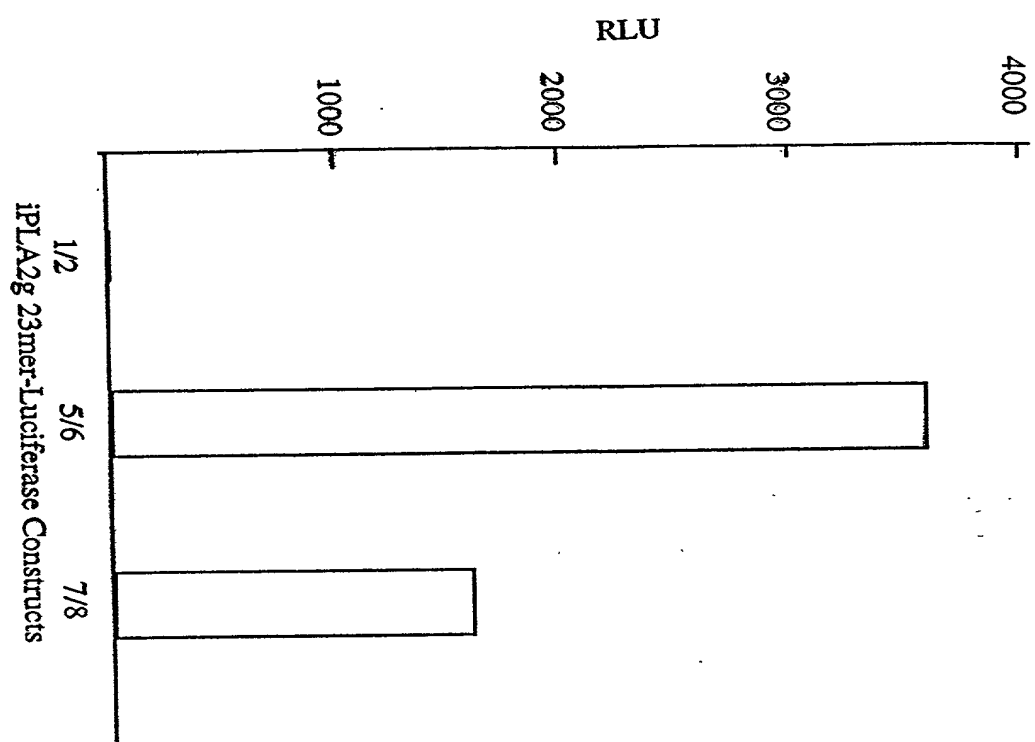


FIG. 24

DECLARATION AND POWER OF ATTORNEY

Attorney's Docket No.
15060-00004

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application and any patent issued thereon.

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